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(54) Title: METHODS AND COMPOSITIONS COMPRISING THE USE OF BLOCKED B–AMYLOID PEPTIDE

(57) Abstract

The present invention relates generally to amyloidogenesis. More particularly, it concerns the use of amyloidogenic proteins that have been blocked at the N– and/or C–termini and/or side chains to prevent their aggregation into amyloidogenic plaques. Particular aspects of the present invention describe the methods and compositions of inhibition of plaque formation in amyloidoses, for example, Alzheimer's Disease.

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DESCRIPTION

METHODS AND COMPOSITIONS COMPRISING THE USE OF BLOCKED B-AMYLOID PEPTIDE

BACKGROUND OF THE INVENTION

This application claims priority to and specifically incoporates by reference, the content of U.S. Provisional Application Serial No. 60/074,658, filed on February 13, 1998.

1. Field of the Invention

The present invention relates generally to fibrillogenesis. More particularly, it concerns the use of blocked Aβ compositions for the inhibition of fibril formation. Particular aspects of the present invention describe the methods and compositions of inhibition of plaque formation in, for example, Alzheimer's Disease.

2. Description of Related Art

Amyloidoses are characterized by the tissue deposition of proteins, which are believed to have a β-sheet conformation in common despite the lack of amino acid sequence homology (Carrel and Lomas, 1997; Horwich and Weissman *et al.*, 1997). Amyloidosis is defined by the deposition of amyloid into tissues of diseases typified by Alzheimer's Disease (AD) and Down's Syndrome. Systemic amyloidoses are characterized by amyloid deposition throughout the viscera. The amyloid of animals is a complex material composed partly of protein fibrils. This protein varies from disease to disease. Among these proteins is Aβ, involved in the pathological progression of Alzheimer's Disease (Glenner and Wong, 1984).

AD is now the fourth-largest killer of adults 65 and older. This disease impacts one of every three families in the United States (Gonzalez-Lima, 1987), and affects over 13 million people world-wide. As the population trends lead to an

increase in the number of older people, this figure will only increase. Thus, it is an important goal of medical science to identify methods of preventing, alleviating or abrogating this disease. The histopathology of AD is characterized by the presence of extracellular plaques and largely intracellular tangles within the cerebral cortex, hippocampus and the diffuse subcortical projection system.

Plaques are made up of a rim of dystrophic neurites surrounding a core of amyloid β-protein formed from abnormally processed amyloid precursor protein (APP). APP is a membrane spanning found in all nerve cells. Tangles occur from abnormally phosphorylated tau protein. Duplication of the APP gene are found in Trisomy 21 (Down's Syndrome; DS) and leads to an Alzheimer's type pathology in the cerebral cortex of individuals with DS (Rosser, 1993).

Aβ is a 40-43 amino acid proteolytic fragment of the transmembrane APP (Kang, 1987; Goldgaber, 1987; Tanzi, 1987). This protein rapidly and irreversibly associates into insoluble fibrils (Kirschner *et al.*, 1987; Hilbich, 1991; Hilbich *et al.*, 1991; Burdick *et al.*, 1992; Castano *et al.*, 1986). The mechanisms of this aggregation, and the structure of the final fibrillar products are not known in detail, however, in this form, the peptides are believed to be neurotoxic. Although these peptides are found in normal brains, they are at higher concentrations in brains from patients with Alzheimer's disease, and these insoluble fibrils are believed to be pathogenic as they form insoluble plaques and tangles in nerves tissues.

It is well recognized in the art that once amyloid deposits have formed, there is no known therapy or treatment which significantly dissolves the deposits *in situ* (US Patent No. 5 ,643,562). Thus, clearly methods and compositions that increase the solubility of the amyloid plaques would be useful in the treatment, prevention and/or inhibition of the progression of pathologies that are characterized by fibrillogenesis and plaque formation.

SUMMARY OF THE INVENTION

Thus, in order to overcome the deficiencies discussed herein above, the present invention provides, in one aspect, a method of inhibiting amyloid fibrillogenesis comprising contacting tissue with a composition comprising an amyloidogenic peptide that has been blocked. In particular embodiments, the peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide. In certain preferred embodiments, the peptide is blocked at the C-terminus of the peptide. In other preferred embodiments, peptide is blocked at the N-terminus of the peptide. In still further embodiments, the peptide is blocked at a side chain of the peptide. In preferred embodiments, the peptide is blocked using a polymer. In certain aspects of the present invention, the polymer is polyethylene glycol.

In certain aspects of the present invention, the amyloidogenic peptide may be selected from the group consisting of cystatin C, AApoA-I, AApoA-II, AScr or PrP-27, Aβ peptide, amyloid A, amyloid kappa L-chain, amyloid lambda L-chain, A β2M, ATTR, AIAPP, amylin atrial naturetic factor, procalcitonin and gelsolin. In other aspects of the present invention the tissue may be selected from the group consisting of pancreas, brain, muscle and heart. In particular aspects, the tissue is from a subject having a pathological state selected from the group consisting of Alzheimer's Disease, Down's Syndrome, Dutch-Type Hereditary Cerebral Hemorrhage Amyloidosis, Reactive (Secondary) Amyloidosis, Familial Mediterranean Fever, Familial Amyloid Nephropathy With Urticaria And Deafness (Muckle-Wells Syndrome), Idiopathic (Primary) Myeloma, Macroglobulinemia-Associated Myeloma, Familial Amyloid Polyneuropathy (Portuguese, Japanese, Swedish), Familial Amyloid Cardiomyopathy (Danish), Isolated Cardiac Amyloid, Systemic Senile Amyloidosis, Adult Onset Diabetes, Insulinoma, Isolated Atrial Amyloid, Medullary Carcinoma Of The Thyroid, Familial Amyloidosis (Finnish), Hereditary Cerebral Hemorrhage With Amyloidosis (Icelandic), Familial Amyloidotic Polyneuropathy (Iowa), Scrapie,

Creutzfeldt-Jacob Disease, Gerstmann-Straussler-Scheinker Syndrome and Bovine Spongiform Encephalitis.

It is contemplated that the subject may be selected from the group consisting of human, sheep and cow. In preferred embodiments, the subject is a human.

Also contemplated by the present invention is a method of inhibiting amyloid fibrillogenesis comprising contacting tissue with a composition comprising a β–amyloid peptide that has been blocked at an end terminal or a side chain. In certain embodiments, the peptide is blocked using a polymer. The polymer may be polyethylene glycol. The Aβ peptide may be blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide. In particular aspects, the Aβ peptide is a full length Aβ peptide. In other embodiments, the Aβ peptide comprises a partial sequence of a full length Aβ peptide. The Aβ peptide may comprise a peptide having amino acid residues 10 through to 35 Aβ peptide. In preferred embodiments, the Aβ peptide has the sequence of SEQ ID NO:1. In other preferred embodiments, the Aβ peptide has the sequence of SEQ ID NO:2.

It is contemplated that the polyethylene glycol has a molecular weight of between about 1000 and 6000 Da. In particularly preferred embodiments, the polyethylene glycol has a molecular weight of about 3000 Da.

Another aspect of the invention provides a Aβ peptide that is blocked by conjugation. In certain embodiments, the Aβ peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide. In particular embodiments, the Aβ peptide is blocked independently at the C-terminus, the N-terminus or a side chain of the peptide. In particular aspects, the Aβ peptide is a full length Aβ peptide. In other aspects, the Aβ peptide comprises a peptide having amino acid residues 10 through to 35 Aβ peptide. In certain embodiments, the peptide is blocked by conjugation to a polymer. In prefered

embodiments, the polymer is polyethylene glycol. In other preferred embodiments, the polyethylene glycol has a molecular weight of about 3000.

The present invention also contemplates a method of inhibiting amyloid plaque formation in a subject comprising administering a pharmaceutical composition comprising a Aβ peptide blocked by conjugation to a second compound and a pharmaceutically acceptable buffer, solvent or diluent. In certain embodiments, the second compound is a polymer. In still other embodiments, the polymer is polyethylene glycol. In certain other embodiments, the Aβ peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide. In preferred aspects, the administering is effected by regional delivery of the pharmaceutical composition. In other aspects, the administering comprises delivering the pharmaceutical composition endoscopically, intratracheally, percutaneously, or subcutaneously. In more particular embodiments, the subject is a mammal. In still further embodiments, the subject is a human. In certain embodiments, the subject suffers from amyloidosis. In particularly preferred embodiments, the subject has Alzheimer's disease. In other preferred embodiments, the subject has Down's Syndrome.

Also provided herein is a pharmaceutical composition comprising a Aβ peptide blocked by conjugation to a second compound and a pharmaceutically acceptable buffer, solvent or diluent. In the pharmaceutical composition, the second compound may be a polymer and the polymer may be polyethylene glycol. In other embodiments, the pharmaceutical composition is defined by having a Aβ peptide that is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various

changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Unlabeled peptides were cross-linked by tTg, then analyzed on Tris-Tricine SDS-PAGE. Cross-linked peptide species ranged from dimeric to hexameric, depending upon the amount of tTg included. In some studies, an extremely faint band consistent with a heptameric structure was seen; however, higher order peptide oligomers could not be observed.

FIG. 2A and FIG. 2B. FIG. 2A: an antiparallel pair of β-strands. FIG. 2B: parallel β-strands showing carbonyl-carbonyl distances consistent with the DRAWS measurement.

FIG. 3A, FIG. 3B and FIG. 3C . Measurable double quantum relaxation exists at both position 15 (Q) and position 16 (K) in the fibrils. FIG. 3A and FIG. 3B are spectra taken of 50 mg 1-^{13}C-K-Aβ fibrils mixed with 10 mg unlabeled HMB. FIG. 3A: Unfiltered CP/MAS spectrum. FIG. 3B: Double quantum filtered spectrum, showing a strong signal for the labeled peptide, but no signal for the unlabeled control, HMB. FIG. 3C: Double quantum efficiency was quantified for 1-^{13}C-Q(15)-Aβ, 1-^{13}C-K(16)-Aβ, the HMB controls, and for ^{13}C-labeled standards, malonic and succinic acid. Comparison of the labeled Aβ results to the standards predicts an interstrand contact with a distance of 5 Å (± 1 Å) for the 1-^{13}C-Q(15)-Aβ and 1-^{13}C-K(16)-Aβ.

FIG. 4. Inter-strand DRAWS data fits 5 Å, 3-spin model. The DRAWS study was run for a series of mixing times (0 to 22 ms). For each mixing time, the carbonyl peak was integrated, normalized to the 0 time point, and adjusted for natural abundance signal to create a decay curve for labeled sample. Data shown are the mean ± SEM (n=5) for 50 mg of 1-^{13}C-Q-Aβ lyophilized fibrils (black circles). Decay curve compared to 4.8 Å (dotted line), 5.0 Å (solid line), and 5.2 Å (dashed line) simulations of 3 spins. For these simulations, it was assumed that each label interacted with 2 other equidistant labels. Decay curve compared to 4.5 Å. (dotted line) and 5.0 A (solid line) simulations for 2 labels in isolation.

FIG. 5. Proposed model of Aβ fibrils. A six-stranded parallel β-barrel, (here, modeled from the core of the tin barrel is consistent with both the enzymatic and NMR data reported in this paper.

FIG. 6A. Preparation of C-PEG-Aβ(10-35). Schematic representation of the C-terminal PEGylation of Aβ(10-35).

FIG. 7A, FIG. 7B and FIG. 7. FIG. 7A. Circular Dichroism Spectroscopy. FIG. 7B. Circular Dichroism Spectroscopy: Circular dichroic (CD) spectra show $[\theta]_{217}$ as functions of pH concentration. FIG. 7C. Circular Dichroism Spectroscopy: Circular dichroic (CD) spectra show $[\theta]_{217}$ as functions of peptide concentration.

FIG. 8A, FIG. 8B and FIG. 8C. FIG. 8A. The amino acid sequence of Aβ can be separated into distinct domains: a hydrophilic N-terminus (aa 1-16), a central hydrophobic region (aa 17-21) and a long hydrophobic C-terminus (aa 29-43). These domains are shown to be conserved in $A\beta_{(10-35)}$ and Aβ-PEG. FIG. 8B. Summary of the inter peptide ^{13}C carbonyl contracts observed for $A\beta_{(10-35)}$ by solid state NMR. Inter peptide distances were measured using DRAWS at positions V_{12}, Q_{15}-V_{18}, V_{24}, G_{26}, G_{29}, G_{33}, and L_{34}. Glycine residues and residues proximal to glycine exhibited larger distances and greater measurement error, ± 0.4 Å, attributable to more flexibility or disorder. Likewise, the amino-terminal V_{12} exhibited considerable

flexibility and a calculated distance of 5.7 ± 0.5Å, but L_{34} showed a well defined contact distance. FIG. 8C. Analysis of the solid state NMR DRAWS study on 1-^{13}C-Leu$_{17}$-Aβ-PEG and 1-^{13}C-Val$_{24}$-Aβ-PEG before and after fibril formation using a series of mixing times from 0 to 22 ms. At each mixing time, the carbonyl peak was integrated and normalized to the first data point (mixing time = 0) to allow comparison between samples. Values shown are the mean ± one standard deviation for 10 mg of lyophilized fibrils (squares.n=5) and 10 mg of non-fibrilized ether precipitate (triangles, n=5) compared to numerical simulations of no interaction (triangles). Simulations are for 5.4 Å (green), 5.2 Å (blue), 5.0 Å (turquoise), 4.8 Å (red), and 4.6 Å (blue) interactions.

FIG. 9A, FIG. 9B, FIG. 9C, FIG. 9D, FIG. 9E and FIG. 9F. Electron Micrographs of $A\beta_{(10-35)}$ titrated with Aβ-PEG at pH 5.7. FIG. 9A. 100%$A\beta_{(10-35)}$. FIG. 9B. 10:1 $A\beta_{(10-35)}$; Aβ-PEG. FIG. 9C. 6:3 $A\beta_{(10-35)}$; Aβ-PEG. FIG. 9D. 1:1 $A\beta_{(10-35)}$; Aβ-PEG; FIG. 9E. 1:4 Aβ-PEG, and FIG. 9F: 100% Aβ-PEG.

FIG. 10A, FIG. 10B and FIG 10C. FIG. 10A. SANS data fit to a modified Guinier plot for a rod-like form of SANS data of $A\beta_{(10-35)}$ and Aβ-PEG in 99% D_2O buffer. The average radii were calculated to be 46 (±5)Å and 65(±1)Å, respectively. FIG. 10B. SANS data fit to a modified Guinier analysis for a rod-like form of $A\beta_{(10-35)}$ and Aβ-PEG in 12% D_2O buffer that eliminates the coherent scattering from PEG. The radii were calculated to be 46 (±5)Å and 47(±5)Å, respectively. FIG. 10C. Schematic representation of the assembly of peptide and PEG in the fibril.

FIG. 11A, FIG. 11B, FIG. 11C and FIG. 11D. Three-dimensional structure of $A\beta_{(10-35)}$. FIG. 11A. Electron Micrograph of a typical paired helical fiber observed for $A\beta_{(10-35)}$ at pH 7.4. FIG. 11B. Enhanced EM showing the paired assembly. FIG. 11C. Structure of the paired fibrils incorporating the 1100 Å repeat composed of 220 β-strands, each with a 1.6° offset to define the twist of the β-helices. Orienting the peptides in parallel and in register would place the hydrophobic C-terminal amino acids entirely along one edge of the β-sheet driving their association. FIG. 11D.

Expansion of the six laminated β-helices using the representation of Cooper (1974). The strands are hydrogen bonded 5 Å from one another within the β-helix propagating with the fibril axis. The amino acid sidechains, oriented perpendicular to the fibril axis, results in the 10 Å separations between the individual sheets.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Alzheimer's disease (AD), the third leading cause of death in the elderly in the US (Hardy and Allsop, 1991), is manifested neuropathologically by dense, extracellular, proteinaceous plaques, composed mainly of the Aβ peptide (Glenner and Wong, 1984). The number of plaques correlates directly with the severity of the disease (Selkoe *et al.*, 1994). The inventors have used DRAWS ^{13}C solid state nuclear magnetic resonance (NMR) studies (Gregory *et al.*, 1997) to measure precise distances between individual Aβ peptides in the plaques. Using synthetic peptides containing a single ^{13}C incorporated at different positions, the inventors have found that the arrangement of adjacent peptides is highly specific, consistent with a model of parallel, but not anti-parallel β-strands aligned precisely at the positions of Glutamine-15 and Lysine-16. These studies demonstrate, for the first time, a generalizable technique by which solid state NMR may be used to determine the three-dimensional structure of Aβ in an amyloid plaque.

From these findings, the inventors developed a series of peptides modified by derivatization or blocking at several loci on the beta amyloid peptides or fragments of these peptides, including the termini and side chain derivatives. In particular, the inventors employed polyethylene glycol to block or modify the side chains and the C- and N-termini of amyloid peptides of varying length. Compared to nonmodified peptides, these PEG-peptides showed enhanced solubility. Interestingly, PEG-beta amyloid peptides showed inhibited fibrillogenesis, suggesting that PEGylated peptides or analogues thereof might be used as inhibitors of fibrillogenesis *in vivo*. Furthermore, the peptides have most of the salient structural features of the nonmodified peptides and therefore can be used as structural probes. For example, C-

terminal modified PEG- Aβ (10-35) shares all known structural features of the nonmodified peptide, yet the former is sufficiently soluble for detail two-dimensional NMR studies that can elucidate the entire structure of the peptide.

Thus in a preferred embodiment, inventors report the synthesis and characterization of plaque-competent Aβ peptides, derivatized at the C-terminus, N-terminus or containing sides chain modification with polyethyleneglycol (PEG). These modifications, while preserving the salient structural features of the non-modified peptide, markedly enhances solubility of Aβ, which can allow for obtaining detailed structural information. Furthermore, the formation of fibrils by Aβ is fully reversible through changes in pH and protein concentration, again in contrast with the nonmodified peptide. The present invention provides a strategy for dissecting the steps in the process of amyloid fibrillogenesis, and may be exploited in the development of site-specific inhibitors of this process. These and other aspects of the present invention are described in further detail herein below.

A. Amyloidosis

Amyloidosis refers to a pathological condition characterized by the presence of amyloid. Amyloid is a generic term referring to a group of diverse but specific extracellular protein deposits which are seen in a number of different diseases. Though diverse in their occurrence, all amyloid deposits have common morphologic properties, stain with specific dyes (*e.g.*, Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. They also share common ultrastructural features and common x-ray diffraction and infrared spectra.

Amyloidosis can be classified clinically as primary, secondary, familial and/or isolated. Primary amyloidosis appears *de novo* without any preceding disorder. Secondary amyloidosis is that form which appears as a complication of a previously existing disorder. Familial amyloidosis is a genetically inherited form found in particular geographic populations. Isolated forms of amyloidosis are those that tend to involve a single organ system. Different amyloids are also characterized by the

type of protein present in the deposit. For example, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease and the like are characterized by the appearance and accumulation of a protease-resistant form of a prion protein (Hope and Baybutt, 1991; Prusiner, 1991; Chesbro, 1991) in the central nervous system.

Similarly, Alzheimer's disease, is characterized by congophilic angiopathy, neuritic plaques and neurofibrillary tangles, all of which have the characteristics of amyloids. In this case, the plaque and blood vessel amyloid is formed by the beta protein. Other systemic diseases such as adult-onset diabetes, complications of long-term hemodialysis and sequelae of long-standing inflammation or plasma cell dyscrasias are characterized by the accumulation of amyloids systemically. In each of these cases, a different amyloidogenic protein is involved in amyloid deposition.

The present invention pertains to methods and compositions useful for treating amyloidosis. The methods of the invention involve administering to a subject a therapeutic compound which inhibits amyloid deposition. More particularly, the present invention involves the use of blocked amyloid peptides so that the aggregation of these peptides into plaques is inhibited. "Inhibition of amyloid deposition" is intended to encompass prevention of amyloid formation, inhibition of further amyloid deposition in a subject with ongoing amyloidosis and reduction of amyloid deposits in a subject with ongoing amyloidosis.

Inhibition of amyloid deposition may be determined relative to an untreated subject or relative to the treated subject prior to treatment. In preferred embodiments, the present invention provides for the inhibition of fibril formation by β–amyloid peptide. Amyloid deposition or fibrillogenesis is inhibited by inhibiting an interaction between monomers of the amyloidogenic protein. In more defined aspects this is achieved by blocking the end termini or side chains of the amyloidogenic protein so that the monomers can not interact. Such "blocking" may be achieved using, for example, PEG.

Thus, the methods of the present invention in a broad aspect are useful for treating amyloidosis associated with any disease in which amyloid deposition occurs. Clinically, amyloidosis can be primary, secondary, familial or isolated. Amyloids have been categorized by the type of amyloidogenic protein contained within the amyloid. Exemplary amyloidogenic proteins that may be modified by the methods of the present invention include those listed in Table 1. The pathological states associated with aberrant expression or mutation in these peptides are also listed.

Other peptides include fibrinogen-associated amyloid and lysozyme-associated amyloid. In particularly preferred aspects, the present invention is directed to the modification of β-amyloid peptides such that the formation of insoluble fibrils is inhibited. The compositions and methods described herein will thus be useful in the inhibition, abrogation or alleviation or the deleterious effects of plaque formation in Alzheimer's disease, Down's syndrome, and Dutch hereditary cerebral hemorrhage amyloidosis. Various peptides are involved in amyloidosis including but not limited to atrial and brain natriuretic peptide (Pucci *et al.*, 1991; Kaye *et al.*, 1986; Benvenga and Facchiano 1995); gelsolin (Steiner *et al.*, 1995; Kivela *et al.*, 1994; Maury *et al.*, 1994) Cystatin (Abrahamson, 1996; Jacobson and Buxbaum, 1991; Bobek *et al.*, 1991 Abrahamson *et al.*, 1990) Apolipoprotein A-I (Johnson *et al.*, 1992 Soutar *et al.*, 1992; Rader *et al.*, 1992) and Apolipoprotein A-II (Higuchi *et al.*, 1991).

Table 1. Amyloidogenic proteins and pathological states associated therewith. Exemplary Genbank sequences for peptides are listed and incorporated herein by reference.

Peptide	Pathology
β-amyloid peptide	Alzheimer's Disease
	Down's Syndrome
	Dutch-Type Hereditary Cerebral
	Hemorrhage Amyloidosis

Table 1 (continued)

Peptide	Pathology
amyloid A (Genbank P22614; P02735; J03474; D32001; A38974; I39456; S48983; X51442)	Reactive (Secondary) Amyloidosis Familial Mediterranean Fever Familial Amyloid Nephropathy With Urticaria And Deafness (Muckle-Wells Syndrome)
amyloid kappa L-chain (Genbank:P04430; P01617; L33854) or amyloid lambda L-chain	Idiopathic (Primary) or Macroglobulinemia-Associated Myeloma
A beta 2M	Chronic Hemodialysis
ATTR	Familial Amyloid Polyneuropathy (Portuguese, Japanese, Swedish) Familial Amyloid Cardiomyopathy (Danish) Isolated Cardiac Amyloid Systemic Senile Amyloidosis
AIAPP (Genbank X68830; P10997) or amylin (Genbank: M27503)	Adult Onset Diabetes Insulinoma
atrial naturetic factor	Isolated Atrial Amyloid
procalcitonin (Genbank P06881)	Medullary Carcinoma Of The Thyroid
gelsolin (Genbank: U28044; M36927; X75629; X75630)	Familial Amyloidosis (Finnish)
cystatin C (Genbank: X52255; M58167, M58169)	Hereditary Cerebral Hemorrhage With Amyloidosis (Icelandic)
AApoA-I (Genbank P02647; J00098)	Familial Amyloidotic Polyneuropathy (Iowa)
AApoA-II	Accelerated Senescence In Mice

Table 1 (continued)

Peptide	Pathology
AScr or PrP-27 (Genbank: P27177; P23907; P13852; P10279; P04925; P04156; S53625; S53624	Scrapie Creutzfeldt-Jacob Disease, Gerstmann-Straussler-Scheinker Syndrome, Bovine Spongiform Encephalitis

B. β-Amyloid Peptide, An Exemplary Amyloidosis Protein

The principal chemical constituent of the amyloid plaques and vascular amyloid deposits (amyloid angiopathy) characteristic of AD and certain the other disorders mentioned above is an approximately 4.2 kilodalton (kD) protein of about 39-43 amino acids designated the β-amyloid peptide (Aβ, or sometimes β–AP, AβP or β/A4. β AP). Aβ was first purified and a partial amino acid sequence reported in Glenner and Wong (1984). The isolation procedure and the sequence data for the first 28 amino acids are described in U.S. Pat. No. 4,666,829. U.S. Patent 4,912,206 describes a cDNA clone for the Alzheimer's Amyloid peptide and the open reading frame of the sequenced clone as well as mapping the gene, which is highly conserved, to human chromosome 21. Both of these patents have been incorporated herein by reference.

Molecular biological and protein chemical analyses have shown that Aβ is a small fragment of a much larger precursor protein, referred to as the β-amyloid precursor protein (APP), that is normally produced by cells in many tissues of various animals, including humans. Knowledge of the structure of the gene encoding APP has demonstrated that Aβ arises as a peptide fragment that is cleaved from APP by as-yet-unknown enzymes (proteases). The precise biochemical mechanism by which the Aβ fragment is cleaved from APP and subsequently deposited as amyloid plaques in the cerebral tissue and in the walls of cerebral and meningeal blood vessels is currently unknown. Several lines of evidence indicate that progressive cerebral

deposition of Aβ plays a seminal role in the pathogenesis of AD and can precede cognitive symptoms by years or decades (for review, see Selkoe, 1991).

An important line of evidence is the discovery in 1991 that missense DNA mutations at amino acid 717 of the 770-amino acid isoform of APP can be found in affected members but not unaffected members of several families with a genetically determined (familial) form of AD (Goate *et al.*, 1991; Chartier Harlan *et al.*, 1991; Murrell *et al.*, 1991) and is referred to as the Swedish variant. A double mutation changing lysine^{595} -methionine^{596} to asparagine^{595} -leucine^{596} (with reference to the 695 isoform) found in a Swedish family was reported in 1992 (Mullan *et al.*, 1992).

Genetic linkage analyses have demonstrated that these mutations, as well as certain other mutations in the APP gene, are the specific molecular cause of AD in the affected members of such families. In addition, a mutation at amino acid 693 of the 770-amino acid isoform of APP has been identified as the cause of the Aβ deposition disease, HCHWA-D, and a change from alanine to glycine at amino acid 692 appears to cause a phenotype that resembles AD in some patients but HCHWA-D in others. The discovery of these and other mutations in APP in genetically based cases of AD proves that alteration of APP and subsequent deposition of its Aβ fragment can cause AD.

As stated earlier Aβ, is an approximately 4.2 kD protein which, in the brains of AD, Down's Syndrome, HCHWA-D and some normal aged subjects, forms the subunit of the amyloid filaments comprising the senile (amyloid) plaques and the amyloid deposits in small cerebral and meningeal blood vessels (amyloid angiopathy). Aβ can occur in a filamentous polymeric form (in this form, it exhibits the Congo-red and thioflavin-S dye-binding characteristics of amyloid described in connection therewith). Aβ can also occur in a non-filamentous form ("preamyloid" or "amorphous" or "diffuse" deposits) in tissue, in which form no detectable birefringent staining by Congo red occurs. U.S. Pat. No. 4,666,829 describes the insoluble form of Aβ. In whatever form, Aβ is an approximately 39-43 amino acid fragment of a large

membrane-spanning glycoprotein, referred to as the β-amyloid precursor protein (APP), encoded by a gene on the long arm of human chromosome 21. Aβ is further characterized by its relative mobility in SDS-polyacrylamide gel electrophoresis or in high performance liquid chromatography (HPLC). Its 43-amino acid sequence is given in SEQ ID NO:1 or a sequence that is substantially homologous thereto.

The Aβ peptide is itself toxic to cultured neurons (Yankner *et al.*, 1989; Yankner *et al.*, 1990; Yankner, *et al.*, 1990; Roher *et al.*, 1991). Several animal models have also been produced in which Aβ is over-produced, though the results of these models have been ambiguous (Yankner, 1996).

The development of Aβ fibrils appears to proceed through one or more soluble, aggregated oligomeric intermediates (Jarrett *et al.*, 1994; Lomakin *et al.*, 1996; Jarrett and Lansbury, 1993). Water soluble Aβ oligomers have been isolated from the media of cultured neuronal cell lines expressing endogenous or transfected βAPP (Seubert *et al.*, 1992; Shoji *et al.*, 1992). Similar or identical soluble oligomers have been isolated from CSF of Alzheimer's Disease patients and normal subjects (Haass *et al.*, 1992; Busciglio *et al.*, 1993; Soto and Castano, 1996).

Samples of CSF from Alzheimer's Disease patients have approximately six times the concentration of oligomers as normal CSF. Quasielectric light scattering confirms the presence of soluble fibrils prior to the formation of insoluble fibrils (Lomakin *et al.*, 1996). The formation of soluble oligomers is accompanied by a conformational transition observable by circular dichroic spectroscopy. The monomeric peptide is initially a so-called "random coil" by CD; as the oligomers form there is an increase in the β content (Soto and Castano, 1996; Soto and Frangione, 1995). Despite the increase in β content, however, the CD curve of the oligomer is not the classic β sheet curve observed by Greenfield and Fasman (Greenfield and Fasman, 1969), *i.e.* with a minimum of ≈ -18,000 deg-cm^2/dmol at 217-218 nm. The secondary structure (as defined by CD) can be altered by changing solvent conditions *e.g.*, the peptide is a helical when dissolved in 50% (v/v) trifluoroethanol (Soto *et al.*,

1995). In several studies, Aβ was neurotoxic only under solvent conditions in which the β sheet structure is present (Buchet *et al.*, 1996; Sato *et al.*, 1995; Li *et al.*, Simmons *et al.*, 1994).

Because of the association with Alzheimer' s Disease, a number of structural approaches have been taken to Aβ. A characteristic finding of Aβ, in common with all other amyloid-forming proteins and is in fact, the amyloid-defining finding, is its ability to bind Congo Red dye which displays green birefringence (Puchtler *et al.*, 1962; Ashburn *et al.*, 1996). Aβ also binds thioflavin S, in common with other amyloids. A mainstay of aggregation studies is the use of turbidimetry to measure solubility changes, and quasielectric light scattering to monitor aggregation in real time.

CD spectroscopy has been used to help define the β sheet-like character of the aggregates. X-ray diffraction studies have also demonstrated a β-stranded structure, but have thus far failed to yield detailed structural information (Kirschner *et al.*, 1986). FTIR has the advantage of being able to study insoluble aggregates, and has also shown β sheet-like structure (Hilbich *et al.*, 1991; Fraser *et al.*, 1991).

EM is widely used, and has demonstrated the regularity and order of the fibrils (Narang *et al.*, 1980, Merz *et al.*, 1983). X-ray crystallography has not yet been used because of the difficulty in obtaining crystals of Aβ. Many of the NMR approaches thus far have been limited to those in which Aβ is highly soluble (Jayawickrama *et al.*, 1995; Sticht, 1995; Kirshenbaum and Daggett, 1995; Talafous *et al.*, 1994; Zagorski and Barrow, 1992; Sormachi and Craik, 1994). Unfortunately, such conditions are probably not relevant to the β sheet-like structure in the fibrils. The structure of Aβ in 60% TFE has been solved, in this solvent it is mainly α helical, which probably reflects more its structure in the neuronal membrane than in the fibril.

A partial structural elucidation of the Aβ (amino acids 1-28) in DMSO has also been reported. This peptide was found to have α helical character; this is in

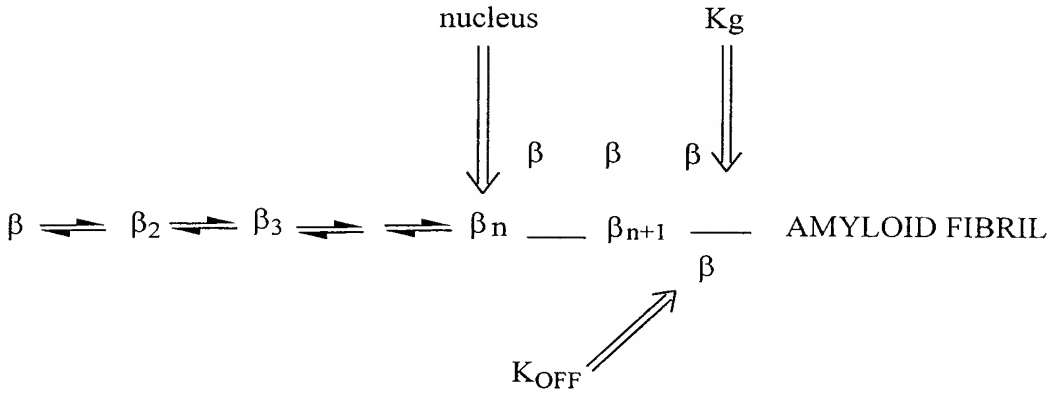
contrast to the inventors' data on Aβ (amino acids 10-35) in DMSO which is highly structured and, importantly, has NOE signatures typical of β strand. In one particularly promising approach, Lee *et al.* (1995) studied a truncated peptide comprised of residues 10-35 of Aβ. In contrast to other truncated peptides, even Aβ (amino acids 1-28), which makes fibrils indistinguishable in EM from those made by full length Aβ, the 10-35 truncation Aβ (amino acids 10-35) was able to add to bona fide Alzheimer's brain plaques when dissolved at peptide concentrations present in CSF.

Therefore, this peptide was chosen for further studies presented herein. Also when dissolved in water at pH 2.1, some of the peptide precipitated, but the remainder stayed in solution indefinitely, *i.e.* for the duration of the NMR studies or longer. NMR data was obtained using a 750 mHz instrument; it showed essentially a β strand-like structure and some NOEs suggestive of a central turn. Because of the limited solubility of this peptide, a full structure determination was not possible. Lansbury *et al.* took a solid state NMR approach towards a C-terminal nonapeptide of Aβ and concluded that it contained a β turn (Lansbury *et al.*, 1995). The inventors have for the first time, definitively characterized the molecular arrangement of Aβ peptide. These results are discussed in relation to plaque formation in AD.

C. Molecular Arrangement of Aβ Peptide in AD

It has been shown, by electron microscopy and x-ray scattering, that Alzheimer's amyloid plaques consist of a highly ordered, fibrillar structure which can be replicated *in vitro* using synthetic peptides. The basic secondary structure of these fibrils is considered to be a β-strand; however, the precise arrangement of the individual peptides in these fibrils has eluded researchers, primarily because the insoluble nature of these fibrils renders them unsuitable for solution NMR or x-ray crystallographic techniques. The inventors have developed a generalizable approach, using solid state NMR and specifically labeled synthetic peptides, by which determination of the specific structure of the peptide in its plaque-conformation is possible.

Jarrett *et al.*, (1994) have proposed the following schema to describe the process of amyloid fibrillogenesis



The schema as written is very general; nevertheless, there are several important questions embedded within it, among them the following. What is the difference between the forms labeled β, β2, β3, *etc.*? What is the structure of the "nucleus", βn? And does growth of the amyloid fibrils actually occur through the addition of one Aβ molecule at a time, or do the βn nuclei aggregate, or both? Is there only a single pathway from β to βn, as depicted in the schema, or are there multiple pathways? What are the energetics of the conversion of β to βn, (*etc.*) and what accounts for the ability of fibrillogenesis to be propagated?

A first step in addressing some of these questions is to define some of these forms structurally. As the preceding questions indicate, the determination of a form does not necessarily mean the determination of the unique form: there may be numerous pathways and structures that lead to fibril formation. In order to increase the likelihood of following a biologically relevant path, the present invention used the truncated form of Aβ described by Lee *et al.*, *i.e.* Aβ(10-35), since this peptide was shown to add to bona fide amyloid plaques from Alzheimer's Disease brains. The inventors' goal was to circumvent the blocks and to determine the structures of some of the forms of Aβ(10-35) in the above pathway. Towards this end, the inventors have identified the molecular arrangement of Aβ in fibrils using crosslinking by tissue

transglutaminases and by NMR studies. The results of these investigations are described herein below and in the Examples.

a. Crosslinking of Aβ(10-35) by tissue transglutaminases

Tissue transglutaminases are able to crosslink full length Aβ at a specific site: Glnl5 to Lysl6 (Dudek and Johnson, 1994; Ikura *et al.*, 1993). The enzyme never uses Lys28 to form the adduct. The inventors have shown that a similar reaction occurs in Aβ(10-35). The inventors have demonstrated that that when the mixture of Aβ and transglutaminase is analyzed bands of 3, 6, 9, 12, 15, and 18 kDa are seen, consistent with monomer, dimer, trimer, tetramer, pentamer, and hexamer.

From these results, the inventors suggest that the transglutaminase links Aβ amino acid side chains that are proximate in space. This was demonstrated by the fact that of the crosslink is exclusively between Glnl5 and Lys16, never between Gln15 and Lys28. Further, the secondary structure most compatible with this crosslink is a β-stranded structure. This is demonstrated by the fact that Glnl5 and Lysl6 are not crosslinked within a single chain, even though the spacing for such a crosslink would be possible in an α helical, β-turn or random coil conformation.

The fibril may be composed of units of 6 Aβ chains, or some multiple of 6 Aβ chains. In contrast, an interdigitating fibril, such as collagen or hemoglobin S, forms a continuous array of higher oligomers upon crosslinking (Adachi *et al.*, 1983; Mirchev and Ferrone, 1997; Rossler *et al.*, 1995; Reiser *et al.*, 1992; Eyre *et al.*, 1984; Bezrukov, 1979). It will be of interest to know the structure of the hexamer, and to test which of the crosslinked aggregates are competent to form fibrils, as judged by EM or binding of dyes like Congo Red or Thioflavin S. If the amyloid fibril is composed of units of 6n chains of Aβ, then the monomer, dimer, trimer, tetramer and hexamer could form fibrils, but the pentamer (not an even divisor of 6) should not. In order to investigate these findings further, the inventors examined Aβ(10-35) in the fibrillar state by solid state NMR spectroscopy as described herein below.

b. Solid State NMR Spectroscopy

The inventors examined Aβ(10-35) in the fibrillar state by solid state NMR spectroscopy. The DRAWS (Dipolar Recoupling with Windowless Sequence) pulse sequence was devised and developed, respectively, by David Gregory (Gregory *et al.*, 1996; Gregory *et al.*, 1997) and Robert Botto. It serves to reintroduce dipolar coupling between two nuclei, and to suppress chemical shift anisotropy (CSA). The goal was to measure dipolar coupling strengths, in this case between ^{13}C nuclei. The sequence reintroduces dipolar coupling with a series of carefully timed pulses during magic angle spinning. The coupling strength is proportional to $1/r^3$ (r = internuclear distance); the maximal distance observed by this method is approximately 6.5 Å.

A 26-amino acid peptide, comprising residues 10-35 of the Aβ peptide was synthesized (SEQ ID NO:2). This peptide was chosen because it has been demonstrated that while it is more soluble, thus much more amenable to laboratory studies, than the native peptide, it still retains the ability to add to authentic plaques isolated from AD patients (Lee *et al.*, 1995). Furthermore, like the native peptide (Dudek and Johnson, 1994), the inventors found that this peptide could be specifically cross linked by the enzyme tissue tranglutaminase (tTg) at the positions of Lys- 15 and Gln-16 of adjacent peptides.

The inventors found that those peptides containing 1-^{13}C at the position of Gln-15 and those containing 1-^{13}C-Lys-16 both had detectable DQ signal. Compared to control samples, this signal strength placed the inter-peptide distance between 4 and 6 Å.

The inventors found the distance for both the 1-^{13}C-Gln-Aβ and 1-^{13}C-Lys-Aβ samples to be 5.0 Å (±0.3); this was true both for repeated measurements of the same sample and for repeated sample preparations. Interestingly, the fit to a 5.0 Å distance was valid only for simulations in which each spin was coupled to 2 other spins at identical distances apart. Models of 2 spins in isolation, at a distance of 4.5 to 5 Å, matched the range of DRAWS data collected, but not the shape of the curve.

Similarly, models of 3 spins where one pair of contacts is close and the second pair is far, such as might be seen in a linear arrangement of 3 spins, failed to fit the data.

Finally, the inventors employed molecular modeling to analyze possible peptide conformations consistent with these distance constraints. Examination of over 20 possible β-strand configurations (parallel, anti-parallel, sheets, barrels, and different alignments of the two strands), revealed, surprisingly, only one possible solution: multiple parallel β-strands aligned at exactly the position of the carbonyls (1-^{13}C) of the glutamines and lysines at positions 15 and 16. The combinations of parallel and antiparallel β-strands, or staggered parallel β-strands were considered. However, the simulations for 2 and 3 spins revealed that not only is the arrangement parallel for both labeled positions, but in both cases the interactions must be repeated over and over again. This is consistent with the hypothesis that core of fibrils is repeated endlessly, perhaps in a circular fashion a predicted by the inventors' enzymatic data and modeled in FIG. 5.

Thus, the inventors have found a specific, repeating alignment of the β-strands in the AD amyloid fibril. The single-label approach employed here greatly simplifies quantum mechanical analysis of the data and allows for accurate solid state NMR studies on repeating, polymeric structures such as the Aβ fibril, and moves the use of solid state NMR of proteins away from theoretical uses in highly specialized laboratories into an area where it can be used as a general technique for anyone interested in structural biology. For the AD amyloid, the inventors have determined the arrangement of a domain of the fibril in the context of the entire fibril. In this manner, it is now possible to continue through the sequence and elucidate the arrangement of the entire structure, thus breaking through one of the major mysteries of Alzheimer's disease.

Generally, fibrillogenesis is the process by which a soluble biopolymer (most commonly a protein) forms an insoluble aggregate. The aggregate is usually

composed of linear, interwound strands, with crosslinks between the strands. Four examples are the following:

1. Collagen fibrillogenesis: Collagen is secreted from fibroblasts, osteoblasts and other cells as a precursor protein called procollagen. Although collagen, like all proteins, is synthesized in a cell, it is made for the production of extracellular matrix; hence, it is secreted from cells and undergoes fibrillogenesis in the extracellular compartment. Most types of procollagen contain three polypeptide chains called pro-α chains. Procollagen undergoes many post-translational modifications in the cell in which it is synthesized, and these include oxidative deamination of select lysine side chains to yield aldehyde functions ("allysines"). Once outside the cell, the procollagen undergoes a series of limited proteolytic steps by which the globular N- and C-terminal domains are removed. This leaves a triple helical tropocollagen molecule which is now able to associate into microfibrils through non-covalent interactions. Within the microfibril, covalent bonds form between select lysine side chains and the allysyl aldehyde functions. The collagen microfibrils then undergo further levels of aggregation into fibrils, composed of multiple, linearly and laterally associated microfibrils; and then into large fibers which make up the strands of tendons, the bone matrix, *etc.* The degree of lateral self-association and covalent sidechain-sidechain crosslinking renders the collagen fibrils and fibers insoluble and relatively resistant to proteolytic enzymes.

2. Fibrin deposition: Fibrinogen is soluble plasma protein involved in blood coagulation. During the formation of a thrombus or blood clot, proteolytic enzymes are activated in the plasma which lead to the activation of thrombin (from prothrombin). Thrombin makes selective proteolytic cleavages in fibrinogen. Fibrinogen is a large rod-shaped protein composed of 6 polypeptide chains ($\alpha 2\beta 2\gamma 2$). The rod-shape of fibrin can be further defined as having three smaller globular domains connected in series by linear connecting domains. Thus, this shape, a "double dumbbell", has a directionality, *i.e.* a "head" and a "tail". Thrombin cleaves the α chains and the β chains in one position each, to liberate two small peptides

called fibrinopeptides A and B, respectively. The remaining large piece of fibrinogen is then called fibrin, and fibrin self-associates into large, insoluble aggregates, called fibrin polymers or fibrin fibrils. The self-association occurs in two dimensions: linear and lateral. The linear self-association makes head-to-tail contacts between adjacent fibrin molecules. These linear aggregates can also associate laterally to make broad fibrils. As with collagen, linear aggregation yields a long, highly asymmetrical, and hence highly viscous solution, but lateral self-association renders the aggregate insoluble. Note that fibrinogen is synthesized in the liver, circulates as a plasma protein (*i.e.* it is extracellular), and then its conversion to fibrin and subsequent steps of fibrillogenesis all occur in the extracellular compartment.

3. Fibers of Hemoglobin S: Sickle cell anemia is a mutation of one amino acid hemoglobin: the 6th amino acid in the two β chains is mutated from Glu to Val. Under low oxygen conditions, Hemoglobin S undergoes a conformational change to a fiber-forming state. The fibers form through self-association of the β chains containing the solvent-exposed hydrophobic Val6 residue. As in the previous two examples, aggregation can occur linearly or laterally. As in the former examples, too, the lateral association yields large insoluble fibrillar aggregates, sometimes called "tactoids". These tactoids are not only insoluble, but rigid and therefore distort and disrupt red blood cells. In this case, all of the steps of fibrillogenesis occur intracellularly, but the end result is the disruption and destruction of the red blood cells. One of the important consequences of sickle cell anemia is hemolysis. In hemolysis, red blood cells are destroyed, and hemoglobin, which is nephrotoxic, is liberated into the circulation.

4. Amyloid fibrillogenesis: The steps of amyloid fibrillogenesis are still being elucidated. Aβ is a series of peptide, 39-43 amino acids in length, derived from a normal transmembrane protein, the Aβ precursor protein (β-APP). A series of proteases, the identity of which is not known, can cleave β-APP to yield Aβ. In the central nervous system, Aβ is found in the cerebrospinal fluid, which is extracellular. In this compartment, Aβ can self-associate to form amyloid fibrils. There are other

pathways by which amyloid fibrils might form as well. For example, Aβ can bind to an extracellular protein found in the cerebrospinal fluid (and also in the blood) called apolipoprotein E. When bound to apolipoprotein E, the Aβ can be internalized by brain cells through any of several receptor proteins. The receptor proteins for apo E include the LDL receptor, the LDL receptor-related protein (LRP), and GP330, among others. Once internalized by these receptors, the apolipoprotein E-Aβ complex enters the endosome and the lysosome, where it is exposed to proteases. According to one hypothesis, the Aβ may get released from the complex and get liberated from the lysosome, and then is free to enter the cytosol where it become neurotoxic. According to another hypothesis, the main site of amyloid fibrillogenesis is extracellular, and the fibrils *per se*, whether complexed to apolipoprotein E or not, are internalized by cells and then become neurotoxic. In other words, the latter hypothesis puts the site of fibrillogenesis as the extracellular compartment.

As to the process of fibrillogenesis of Aβ itself, it seems that Aβ itself is found in the cerebrospinal fluid (*i.e.* extracellularly) as a free peptide or extremely limited solubility; and that this peptide rapidly self-associates into insoluble fibrils. The aggregation, as in the previous examples, occurs in two dimensions: linearly and laterally. The inventors studies work on PEG-Aβ(10-35) presented herein demonstrate that the linear association generates long, viscous aggregates that are soluble, but the lateral association renders these aggregates insoluble.

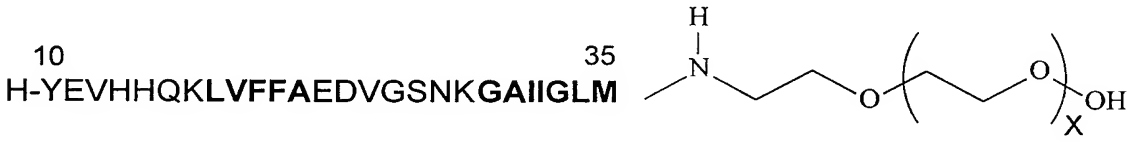
D. Solubilization of Fibrils According to the Present Invention

The present invention provides compositions of soluble peptide derivatives of amyloidogenic peptides. In particular embodiments, the present invention shows that modification of the amyloidogenic peptide to render the side chains and end termini of the peptide unavailable for polymerization results in the generation of modified peptides that act as inhibitors of amyloidogenesis.

The initial impetus of this design was to determine whether the C-terminal and N-terminal of the Aβ peptide were involved in fibril formation. A test of this model,

and a potentially expedient way to obtain detailed structural data would be to derivatize the termini of Aβ in such a way that linear aggregation is blocked by steric hindrance. This was accomplished through use of polyethylene glycol derivatization of N- or C-termini of Aβ (10-35). In an exemplary embodiments, the inventors have developed a series of peptides modified by PEG derivatization at several loci on the beta amyloid peptides or fragments of these peptides, including the termini and side chain derivatives. Compared to nonmodified peptides, PEG-peptides show enhanced solubility. PEG-Aβ peptides show inhibited fibrillogenesis, suggesting that these peptides or analogues thereof will be useful as inhibitors of fibrillogenesis *in vivo*. Furthermore, the peptides have most of the salient structural features of the nonmodified peptides and therefore can be used as structural probes, for site-directed delivery of medicaments.

The Aβ peptide contains a hydrophilic N-terminus, a central hydrophobic region (aa 17-21) and a long and very hydrophobic C-terminus (aa 29-43). Residues 10-35 represents an apparent minimal structure competent to add to bona fide plaques from brains of patients with Alzheimer's Disease (Lee *et al.*, 1995). The inventors have used solid state NMR to demonstrate that the central hydrophobic region forms a parallel β-sheet. Hypothesizing that the C-terminal hydrophobic region mediates an irreversible step in fibril formation, the inventors synthesized the C-terminal PEG-derivatized peptide **1**:



1

From pH 3-7, **1** gave clear solutions while the native peptide under similar conditions of pH, ionic strength and concentrations formed gels or precipitates. There was a dramatic pH induced transition from random coil to a β sheet. Further, there was a modal increase in β content (decreasing $[\theta]_{217}$ from pH 2.7 to 4.5, followed by

the apparent loss of β content due to precipitation of peptide from pH 4.5 to 7.6. From the pH dependency curve, the inventors calculated two pKs, 3.94 and 6.96, perhaps indicating interactions between E11 and H13 in a parallel β sheet, stabilizing the transition to β sheet and formation of aggregates at higher pH. There was an equally marked concentration induced transition, analysis of which was consistent with a reversible monomer-oligomer equilibrium; curve-fitting suggested a hexameric or heptameric species.

Both PEGylated and nonmodified Aβ were crosslinked by tissue transglutaminase, an enzymatic probe of side chain proximity. Crosslinking yielded similar banding "ladders", from monomer to hexamer species on SDS-PAGE, probably between Q15 and K16 as was shown for full length Aβ (Spera and Bax, 1991; Wishart *et al.*, 1992). As expected the molecular weight for each band of **1** was higher than the corresponding band for nonmodified Aβ.

From the data presented in the examples, the inventors propose the following scheme for fibrillogenesis:

$$U \rightleftharpoons Ext_{monomer} \rightleftharpoons \beta_{aggregate} \rightleftharpoons (\beta_{fibril})_{sol} \rightarrow (\beta_{fibril})_{precip}$$

The scheme is somewhat complex, but each step is necessitated by the above data. At low peptide concentrations, low pH, and/or low ionic strength, the peptide is unstructured ("U"; NMR, CD data). The peptide first attains an extended ("$Ex_{monomer}$") but not a classic β-strand conformation as the peptide concentration, pH or ionic strength is raised (2D ^{1}H-NMR data). At still higher peptide concentrations, the classic CD spectrum of a β sheet could be seen, and in the NMR this was associated with the development of line broadening. The peptide appears to aggregate first into a small oligomer ($\beta_{aggregate}$), possible a hexamer (CD, transglutaminase data). Under conditions in which the CD data clearly show that the peptide to be both β sheet and soluble, small, thin and notably not laterally aggregated fibrils are present by EM; the inventors designated this species as a soluble fibril, "$(\beta_{fibril})_{sol}$". The last

transition in the above scheme is the conversion of the soluble fibril to the insoluble fibril (designated "$(\beta_{fibril})_{insol}$" above). The lateral aggregation of linear Aβ fibrils, akin to crosslinking of linear polymers, is inhibited by addition of the C-terminal PEG moiety, as clearly seen on EM. By inhibiting lateral association of the linear aggregates, precipitation of C-PEG Aβ is extremely slow and limited. That is, the C-terminal modification inhibits the last step of the above scheme, accounting for the apparent reversibility of the aggregation.

The data presented herein provide for the first time a congener of the pathogenic Aβ peptide which forms native fibrils in a reversible manner and allow structural events of fibrillogenesis to be characterized in detail. Such modifications can be used to probe regional peptide-peptide interactions, and furthermore may lead to the development of inhibitors of different phases of the fibrillogenesis process. Structural proposals such as those made above can now be defined in this and other amyloidogenic peptides in the early events in nucleation and propagation of fibrils so critical in many disease states.

a. PEGylation to increase solubility

Polyethyleneglycol derivatization has been used to increase the solubility of proteins, often without detectable alterations in tertiary structure (Rajasekharan and Mutter, 1981; Inada *et al.*, 1995; Fortier, 1994; Inada *et al.*, 1994). Therapeutically, PEG-proteins, such as PEG-adenine deaminase, are used because they are functional, and yet have an increased half-life in the plasma (Hershfield *et al.*, 1992; Weinberg *et al.*, 1993; Bax *et al.*, 1996; Hershfield , 1995; Hershfield , 1993). The inventors hypothesized that PEGylation of Aβ might increase the solubility N-terminal PEG-Aβ (10-35), and/or retard fibrillogenesis, while retaining many or all of the structural features of the native peptide.

The inventors synthesized Aβ (10-35) derivatized with PEG on the N- or C-terminus. Further, derivatives containing PEG on both termini of Aβ (10-35) are also contemplated. The PEG- Aβ may comprise the full length sequence of Aβ or

comprise a portion of the Aβ as exemplified by the Aβ (10-35) peptide as used in certain examples herein. It should be understood that mutants of the full length or truncated Aβ can also be used the only limiting factor for the purposes of the present invention is that that Aβ is recognizable as a plaque-competent Aβ. That is to say in the absence of the solubilization by conjugation to PEG the Aβ used will form fibrils and/or plaques.

i. N-terminal Modification

The solubility of N-terminal PEG- Aβ is more than an order of magnitude greater than the underivatized peptide. Despite this dramatic increase in solubility, preliminary NMR data demonstrated that these peptides have NOEs characteristic of an extended structure, and quite similar to those the inventors have measured of Aβ (10-35) in DMSO and to those reported by Lee *et al.,* (1995) for Aβ (10-35) in acidic media. Increases in turbidity over time are also dramatically retarded, with barely any increase observable after a week.

ii. C-terminal Modifications

The synthesis and characterization of a plaque-competent Aβ peptide, Aβ (10-35), derivatized at the C-terminus with polyethyleneglycol (C-PEG-Aβ) was also accomplished herein. This modification, while preserving the salient structural features of the non-modified peptide, markedly enhanced solubility of C-PEG-Aβ. Furthermore, the formation of fibrils by C-PEG-Aβ was fully reversible through changes in pH and protein concentration, again in contrast with the nonmodified peptide. Modification of Aβ at its C-terminus results in a peptide which undergoes at dramatic transition, observable by CD spectroscopy, from random coil to soluble β sheet as the pH is increased from 3.0 to 7.4, or as the peptide concentration is raised to 3.8 mM at pH 5.8.

Solution phase 2-D NMR confirmed a transition from a soluble extended conformation to an oligomeric species, and demonstrated the structural similarity between the modified and unmodified peptides. Electron microscopy demonstrated

that C-PEG modification specifically inhibits the lateral aggregation of fibrils into bundles, but not the longitudinal growth of fibrils, indicating participation of the C-terminus in the transition from soluble to insoluble β sheet.

iii. Additional Modifications

As stated earlier, given the methods and compositions described herein the full structure of Aβ (10-35) derivatized on the N- and/or C-terminal with polyethylene glycol (PEG) is now possible. Given the role of these compounds in the inhibition of fibrillogenesis in AD cells the increased temporal stability and solubility have clear advantages.

The present invention suggests that derivatization of Aβ (10-35) at either terminus would retard or prevent longitudinal aggregation, but not lateral aggregation. Side chain PEGylated Aβ (10-35), for example, derivatized on the e-amino group of Lys16, might then interfere with lateral aggregation. The inventors observed, further, that some aggregation of N-PEG- Aβ (10-35) occurred at neutral pH, but not at pH 3.2. It may be that lateral aggregation does still occur in this peptide, though it can be controlled by the pH.

The rate of fibril formation by native Aβ peptides depends on chain length, *i.e.* longer peptide = more hydrophobic C-terminal portion = more amyloidogenic (Jarrett *et al.*, 1994; Soreghan *et al.*, 1994; Jarrett *et al.*, 1993). The one exception proves the rule, *i.e.* Aβ (1-42) > Aβ (1-43), but residue 43 is the hydrophilic residue, Thr. The formation of oligomers and/or higher aggregates may proceed through interactions between C-terminal domains. Accordingly, steric hindrance of these interactions may be best at retarding polymerization of soluble oligomers into fibrils.

The data suggest that the ability of PEG- Aβs to remain in solution may be related to steric hindrance in either the aggregation of small oligomers into polymers, or the growth (one molecule at a time) of oligomers into fibrils. In either case, the PEG- Aβs may be stuck in the stage of small oligomers.

iv Synthesis of N- and C-PEG- Aβ *(10-35)*

The synthesis of N-terminal and C-terminal PEG- Aβ (10-35) employs techniques well known to those of skill in the art N-terminal PEG- Aβ (10-35) was synthesized by the manual addition of carboxy-PEG to deprotected Aβ (10-35) still attached to the resin. Coupling was carried out by the repeated addition of carboxy-PEG + 4 equivalents of BOP reagent, until manual qualitative ninhydrin test revealed no remaining free amines.

The amino acid sequence is designated as SEQ ID NO:2. Compound **1,** shows the C-PEG- Aβ (10-35) synthesized herein. Compound **1** was synthesized using standard FMOC protocols on PAP Tenta-Gel purchased from Rapp Polymer. PAP resin is cleaved by TFA yielding a linear polyethylene glycol 3000 average molecular weight covalently bound to the carboxyl terminus of the peptide (Bayer and Rapp, 1992; Rapp *et al.*, 1990). The last 10 residues were double coupled to an overall crude coupling yield of 96%. After cleavage from the resin and side-chain deprotection, the peptide had an modal molecular weight of 6212 (2900 peptide + 3000 ave. mol. wt. PEG) by MALDI-TOF mass spectroscopy, was a single peak by RP-HPLC, and one band by SDS-PAGE.

E. Expression of Polypeptides

Particular aspects of the present invention will require the derivatization of amyloidogenic peptides as described above. Such peptides include but are not limited to Aβ peptide, amyloid A, amyloid kappa L-chain, amyloid lambda L-chain, Aβ 2M, ATTR, AIAPP, amylin, atrial naturetic factor, procalcitonin, gelsolin, cystatin C, AApoA-I, AApoA-II, and AScr or PrP-27. These peptide are well known to those of skill in the art and readily commercially available. However, in particular embodiments, it may be necessary to produce recombinant proteins that have a particular sequence or mutation therein. As such, the gene encoding such an amyloidogenic protein can be inserted into an appropriate expression system. The gene can be expressed in any

number of different recombinant DNA expression systems to generate large amounts of the polypeptide product, which can then be purified and used as described herein.

In one embodiment, amino acid sequence variants of a polypeptide can be prepared. These may, for instance, be minor sequence variants of a polypeptide that arise due to natural variation within the population or they may be homologues found in other species. They also may be sequences that do not occur naturally but that are sufficiently similar that they function similarly and/or elicit an immune response that cross-reacts with natural forms of the polypeptide. Sequence variants can be prepared by standard methods of site-directed mutagenesis such as those described below in the following section.

Amino acid sequence variants of a polypeptide can be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the native protein which are not essential for function or immunogenic activity, and are exemplified by the variants lacking a transmembrane sequence described above. Another common type of deletion variant is one lacking secretory signal sequences or signal sequences directing a protein to bind to a particular part of a cell.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide such as stability against proteolytic cleavage. Substitutions preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

Insertional variants include fusion proteins such as those used to allow rapid purification of the polypeptide and also can include hybrid proteins containing sequences from other proteins and polypeptides which are homologues of the polypeptide. For example, an insertional variant could include portions of the amino acid sequence of the polypeptide from one species, together with portions of the homologous polypeptide from another species. Other insertional variants can include those in which additional amino acids are introduced within the coding sequence of the polypeptide. These typically are smaller insertions than the fusion proteins described above and are introduced, for example, into a protease cleavage site.

In one embodiment, major amyloidogenic determinants of the polypeptide are identified by an empirical approach in which portions of the gene encoding a given polypeptide are expressed in a recombinant host, and the resulting proteins tested for their ability to elicit fibrillogenesis, amyloidogenesis or plaque formation. For example, PCR™ can be used to prepare a range of cDNAs encoding peptides lacking successively longer fragments of the C-terminus of the protein (PCR™ technology is disclosed in U.S. Patent 4,683,202 and U.S. Patent 4,682,195, each incorporated herein by reference). The amyloidogenic activity of each of these peptides then identifies those fragments or domains of the polypeptide that are essential for this activity. Further studies in which only a small number of amino acids are removed at each iteration then allows the location of the amyloidogenic determinants of the polypeptide.

Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson *et al.*, (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

Successful applications of the peptide mimetic concept have thus far focused on mimetics of β-turns within proteins. Likely β-turn structure within a polypeptide can be predicted by computer-based algorithms. Once the component amino acids of the turn are determined, peptide mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

Modification and changes may be made in the structure of a gene and still obtain a functional molecule that encodes a protein or polypeptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by change the codons of the DNA sequence, according to the following data.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982).

TABLE 2

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are:

Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

F. Formation of Genetic Constructs

DNA expression plasmids may be designed to optimize production of the heterologous amyloidogenic proteins and mutants thereof described in the previous section. These expression plasmids include a number of enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in cells. Elements designed to optimize messenger RNA stability and translatability in cells are defined.

a. Vector Backbone

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In

certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter

elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid encoding a particular gene is not believed to be important, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the gene of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a gene of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of the gene product following transfection can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product. Tables 3 and 4 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of the gene of interest. This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 3 and Table 4). Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 3

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
β-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein
τ-Globin
β-Globin
e-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)

TABLE 3 (continued)

ENHANCER
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 4

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone
Insulin E Box	Glucose

In preferred embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells *via* receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian

virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kB of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

b. Other Regulatory Elements

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. The inventors have employed the human Growth Hormone and SV40 polyadenylation signals in that they were convenient and known to function well in the target cells employed. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

c. Selectable Markers

In certain embodiments of the invention, the delivery of a nucleic acid in a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding

a gene product. Further examples of selectable markers are well known to one of skill in the art.

G. Delivery of Expression Constructs

In order to effect expression of gene constructs, the expression construct must be delivered into a cell. For protein production, this delivery may be accomplished *in vitro*, in laboratory procedures for transforming cells lines. One mechanism for delivery is *via* viral infection where the expression construct is encapsidated in an infectious viral particle.

Thus, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells *via* receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kb of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

a. Adenovirus

One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide that has been cloned

therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5′-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

Generation and propagation of replication deficient adenovirus vectors, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.,* 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.,* 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Recently, Racher *et al.,* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml

Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.*, (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes

delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.,* 1963; Top *et al.,* 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.,* 1991; Gomez-Foix *et al.,* 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.,* 1990; Rich *et al.,* 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.,* 1991; Rosenfeld *et al.,* 1992), muscle injection (Ragot *et al.,* 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.,* 1993).

b. Retrovirus

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging

cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes *via* sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of

wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact- sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.,* 1988; Hersdorffer *et al.,* 1990).

c. Additional Viral Vectors

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.,* 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.,* 1988; Horwich *et al.,* 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.,* 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.,* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was co-transfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.,* 1991).

d. Non-viral Vectors

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In yet another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well (Dubensky *et al.*, 1984).

In still another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, *i.e. ex vivo* treatment. Again, DNA encoding a particular gene may be delivered *via* this method and still be incorporated by the present invention.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.*, (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*,

then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.,* 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.,* 1993; Perales *et al.,* 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

e. Cell Culture

Primary mammalian cell cultures used for the production of amyloidogenic proteins may be prepared in various ways. In order for the cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

One embodiment of the foregoing involves the use of gene transfer to immortalize cells for the production of proteins. The gene for the protein of interest may be transferred as described above into appropriate host cells followed by culture

of cells under the appropriate conditions. The gene for virtually any polypeptide may be employed in this manner. The generation of recombinant expression vectors, and the elements included therein, are discussed above. Alternatively, the protein to be produced may be an endogenous protein normally synthesized by the cell in question.

Examples of useful mammalian host cell lines are Vero and HeLa cells and cell lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, NIH3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and process the gene product in the manner desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to insure the correct modification and processing of the foreign protein expressed.

Animal cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.* a monolayer type of cell growth).

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. However, suspension cultured cells have limitations, such as tumorigenic potential and lower protein production than adherent T-cells.

Large scale suspension culture of mammalian cells in stirred tanks is a common method for production of recombinant proteins. Two suspension culture reactor designs are in wide use - the stirred reactor and the airlift reactor. The stirred design has successfully been used on an 8000 liter capacity for the production of interferon. Cells are grown in a stainless steel tank with a height-to-diameter ratio of

1:1 to 3:1. The culture is usually mixed with one or more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easily, has good mass transfer of gases and generates relatively low shear forces.

H. Purification of Proteins

Particular aspects of the present invention will employ protein purification techniques to isolate amyloidogenic proteins fro use in the present invention. Such proteins may be recombinant proteins produced as described herein above, or they may be isolated from natural sources. Protein purification techniques are well known to those of skill in the art. These techniques tend to involve the fractionation of the cellular milieu to separated the amylin form other components of the mixture. Having separated amyloidogenic proteins from the other plasma components the amyloidogenic peptide sample may be purified using chromatographic and electrophoretic techniques to achieve complete purification. Analytical methods particularly suited to the preparation of a pure peptides are ion-exchange chromatography, HPLC, FPLC and the like. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al., Biochem. Biophys. Res. Comm., 76*:425, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain and adequate flow rate. Separation can be accomplished in a matter of minutes, or a most an h. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material

is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Conconavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The methods for the generation of antibodies that would be suitable for use in accord with the present invention are well known to those of skill in the art. The skilled artisan is referred to various standard text such as "Antibodies: A Laboratory Manual" (Cold Spring Harbor Laboratory, 1988), Goding, 1986, , and Campbell, 1984, each incorporated herein by reference; as well as U.S. Patent

4,196,265, (incorporated herein by reference) for details regarding antibody production.

I. Monitoring Fibrillogenesis

In particular aspects of the present invention, it may be necessary to test the fibrillogenesis inhibiting or amyloidogenesis inhibiting property of an amyloidogenic peptide modified according to the methods presented herein. As stated earlier, Aβ is an exemplary amyloidogenic peptide and is responsible for amyloid deposits in small cerebral and meningeal blood vessels (amyloid angiopathy). In particular aspects of the present invention has provided compositions of modified Aβ that inhibit fibrillogenesis and in a broader aspect amyloidogenesis. Amyloidogenesis may be monitored using an assay that employs dyes such as Congo-red and thioflavin-S dye because amyloidogenic peptides in their aggregated form become stained whereas those in their soluble form do not become stained.

Therefore, within certain embodiments of the invention, methods are provided for screening for inhibitors of amyloidogenesis. Such methods may use cells that comprise an amyloidogenic phenotype either as adherent cells on a culture dish, as part of an alginate biomatrix, in suspension culture or in any other form that permits the polypeptide form amyloid plaques and for such formation to be monitored for example using staining with the dyes discussed above. These cells are then used as reagents to monitor the effects of the fibrillogenesis inhibiting compositions produced by the present invention.

The present invention provides methods of screening for inhibitors of amyloidogenesis, by monitoring amyloidogenic function in the absence of the candidate substance and comparing such results to the assay performed in the presence of candidate amyloidogenesis inhibitors.

In certain embodiments, the present invention concerns a method for identifying such inhibitors. It is contemplated that this screening technique will prove

useful in the general identification of a compound that will serve the purpose of decreasing, inhibiting or otherwise abrogating the formation of amyloidogenic plaques and fibrils secretion of, for example, any amyloidogenic polypeptide that has been modified so that the C-terminal, the N-terminal and the side chains thereof are not free to form aggregates as described according to present invention.

Such compounds will be useful in the treatment of various amyloidoses resulting from the deposition of an amyloidogenic peptide, such as for example, Alzheimer's Disease, Downs Syndrome, and other amyloidoses for example those listed in Table 1.

In these embodiments, the present invention is directed to a method for determining the ability of a candidate PEGylated amyloidogenic peptide to inhibit the amyloidogenesis around cell that is either naturally amyloidogenic or has been engineered to possess amyloidogenic properties for screening purposes as described herein. Thus as used herein below "a sample having amyloidogenic plaques" may be an adherent cell that has the ability to form amyloid plaques or fibril aggregates. In an alternative embodiment, it is known that amyloidogenic peptides are insoluble in aqueous solution. Thus a simpler assay format that may be employed to test whether a composition is capable of inhibiting fibril formation may comprise a solution of amyloidogenic peptide. Thus in these assays "a sample having amyloidogenic plaques" comprises a solution of amyloidogenic peptide in which amyloidogenesis is exhibited as turbidity of the sample.

The method including generally the steps of:

(a) providing a sample having amyloidogenic plaques;
(b) contacting said sample with a PEGylated amyloidogenic peptide;
(c) measuring the amyloid plaques of said sample; and
(d) comparing the amyloidogenic plaques of the sample in step (c) with the amyloidogenic plaques of the sample of step (a).

To identify a candidate substance as being capable of inhibiting amyloid plaque formation in the assay above, one would measure or determine the amyloid plaque formation in the absence of the added candidate substance by determining for example the turbidity of the solution, or the presence of the number of plaques and such. One would then add the candidate substance to the cell and determine the amyloidogenesis in the presence of the candidate substance. A candidate substance which decreases the amyloidogenesis or fibril forming capacity relative to the function in its absence, is indicative of a candidate substance with inhibitory capability.

Fibrillogenesis is the process by which fibrils appear in aggregates the extracellular matrix of tissue. In solution fibrillogenic aggregates increase the turbidity of a solution. The presence of these aggregates may be monitored and quantified using any of a number of techniques. For example, the turbidity of a solution can be measured spectrophotometrically, the presence of aggregates may be measured and monitored using microscopy and amyloidogenesis can be monitored using ELISA based techniques that employ dyes such as Congo red for the detection of plaque forming peptides. Further, US Patent 5,538,845, is incorporated herein by reference, in that it provides methods for identifying compounds capable of inhibiting the production of Aβ peptide in cells. Such methods may be used in conjunction with the Aβ inhibitors generated herein to assess the ability of such compound to inhibit wild-type Aβ function in plaque formation.

U.S. Patent 5,474,893, incorporated herein by reference, describes a spectrophotometric method for measurement of amyloid degrading activity in serum or tissue. The method involves first binding a dye to a purified amyloid protein. Preferably, the dye is Congo red. The composition comprising a dye bound to a purified amyloid protein is mixed with serum or tissue homogenate to form a reaction mixture. Thus, the activity of the fibrillogenesis inhibiting compositions of the present invention may also be monitored using the methods set forth in U.S. Patent

5,474,893, wherein degradation of the amyloid plaque by the inhibitors produced herein will disrupt the dye/amyloid bond causing dye to be released into a supernatant solution where its concentration and amount are spectrophotometrically determined. The amount of the amyloid degraded is calculated based upon the quantity of the dye found in the supernatant. U.S. Patent 5,348,963 also provides methods that may be useful in combination with the present invention when screening for inhibitors of amyloid formation.

Fibril formation may also be monitored using HPLC. In such a method the disappearance of the monomer peak is indicative of aggregation. This may also be confirmed by electron microscopy. This HPLC method to measure fibrillogenesis uses light scattering. Examples of the use of quasielastic light scattering to measure fibrillogenesis of Aβ has been extensively studies and is well known to those of skill in the art (Walsh *et al.*, 1997; Wood *et al.*, 1996; Lomakin *et al.*, 1997; Wood *et al.*, 1996; Lomakin *et al.*, 1996; Shen *et al.*, 1993).

In the present invention this technique has been used in the following procedure: High performance size exclusion chromatography with multiangle light scattering detector (HPSEC-MALLS) is performed using the Showa Denko Shodex Protein KW-804 size exclusion column, on a Wyatt Technologies DAWN Laser photomer/OPTILAB refractometer system The molecular weight is calculated using the procedure described in the Wyatt Technologies manual using identical conditions of pH, ionic strength and peptide concentration as in the CD analysis. In addition to light scattering, scattering of other types of radiation can be used for similar purposes, *e.g.*, small angle X-ray diffraction or small angle neutron scattering.

As used herein the term "candidate substance" refers to any molecule that is capable of inhibiting amyloidogenic function. In particular embodiments, the candidate substance may be a protein or fragment thereof, that has been modified to prevent the formation of aggregates. It may prove to be the case that the most useful pharmacological compounds for identification through application of the screening

assay will be compounds that are structurally related to other known amyloidogenic peptides. The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such compounds in humans or animal models, it will be necessary to test a variety of candidates to determine which have potential.

The candidate screening assays are simple to set up and perform. Thus, in assaying for a candidate substance, after obtaining an amyloidogenic sample, one will admix a candidate substance with the sample, under conditions which would allow measurable amyloidogenesis to occur. In this fashion, one can measure the ability of the candidate substance to inhibit the amyloidogenic parameter in the absence of the candidate substance. In this fashion the ability of the candidate inhibitory substance to reduce, abolish, or otherwise diminish amyloidogenesis may be detected.

"Effective amounts" in certain circumstances are those amounts effective to reproducibly inhibit amyloidogenesis or fibril formation in comparison to their normal levels. Compounds that achieve significant appropriate changes in activity will be used.

Significant decreases in amyloidogenesis or fibril formation, *e.g.*, as measured using spectrophotometry, ELISA, microscopy and the like are represented by a decrease in fibril formation of at least about 30%-40%, and most preferably, by decreases of at least about 50%, with higher values of course being possible.

J. Pharmaceutical Compositions

The present invention further comprises pharmaceutical compositions incorporating a PEGylated amyloidogenic compound described herein above and including a pharmaceutically acceptable carrier. Such pharmaceutical compositions should contain a therapeutic or prophylactic amount of at least one compound identified by the method of the present invention.

Thus, the phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The pharmaceutical compositions just described are suitable for systemic administration to the host, including both parenteral, topical, and oral administration. The pharmaceutical compositions may be administered parenterally, *i.e.* subcutaneously, intramuscularly, or intravenously. Thus, the present invention provides compositions for administration to a host, where the compositions comprise a pharmaceutically acceptable solution of the identified compound in an acceptable carrier, as described above. Typically, injectibles are prepared either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

The pharmaceutical forms suitable for injectible use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectible solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Sterile injectible solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectible solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparation of more, or highly, concentrated solutions for local injection also is contemplated. In this regard, the use of DMSO as solvent is preferred as this will result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms

Formulations of neutral or salt forms are also provided. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The pharmaceutically acceptable carrier can be any compatible, non-toxic substance suitable to deliver the compounds to an intended host. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectible compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Pharmaceutically acceptable adjuvants, buffering agents, dispersing agents, and the like may also be incorporated into the pharmaceutical compositions. Preparation of pharmaceutical conditions incorporating active agents is well described in the medical and scientific literature. See, for example, Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 16th Ed., 1982, the disclosure of which is incorporated herein by reference. Dosages may vary according to the physician's diagnosis and the route of administration chosen. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated or diagnosed. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

Under ordinary conditions of storage and use, the cell preparations may further contain a preservative to prevent growth of microorganisms. Intravenous vehicles

include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components in the pharmaceutical are adjusted according to well-known parameters.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is diagnostically or therapeutically effective. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In other embodiments, direct injection into the plaque is contemplated. Alternatively, the region of the amyloid plaque may be infused or perfused with the therapeutic compounds using any suitable delivery vehicle. Local or regional administration, with respect to the plaque, also is contemplated. Finally, systemic administration may be performed. Continuous administration also may be applied where appropriate. Delivery *via* syringe or catherization is also contemplated.

Frequently, it will be desirable or necessary to introduce the pharmaceutical compositions directly or indirectly to the brain. Direct techniques usually involve placement of a drug delivery catheter into the host's ventricular system to bypass the blood-brain barrier. Indirect techniques, which are generally preferred, involve formulating the compositions to provide for drug latentiation by the conversion of hydrophilic drugs into lipid-soluble drugs. Latentiation is generally achieved through blocking of the hydroxyl, carboxyl, and primary amine groups present on the drug to render the drug more lipid-soluble and amenable to transportation across the blood-brain barrier. Alternatively, the delivery of hydrophilic drugs can be enhanced by intra-arterial infusion of hypertonic solutions which can transiently open the blood-brain barrier.

The concentration of the pharmacologically active compound in the pharmaceutical carrier may vary widely, *i.e.* from less than about 0.1% by weight of the pharmaceutical composition to about 20% by weight, or greater. Typical pharmaceutical composition for intramuscular injection would be made up to contain, for example, one to four ml of sterile buffered water and one μg to one mg of the compound identified by the method of the present invention. The typical composition for intravenous infusion could be made up to contain 100 to 500 ml of sterile Ringer's solution and about 1 to 100 mg of the compound.

The pharmaceutical compositions of the present invention can be administered for prophylactic and/or therapeutic treatment of diseases related to the deposition of Aβ and other amyloidogenic peptides, such diseases including but not limited to Alzheimer's disease, Down's syndrome, and advanced aging of the brain. In therapeutic applications, the pharmaceutical compositions are administered to a host already suffering from the disease. The pharmaceutical compositions will be administered in an amount sufficient to inhibit further deposition of Aβ plaque. In other embodiments, the size of the pre-existing amyloid plaque is diminished. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Such effective dose will depend on the extent of the disease, the size of the host, and the like, but will generally range from about 0.01 μg to 10 mg of the compound per kilogram of body weight of the host, with dosages of 0.1 μg to 1 mg/kg being more commonly employed.

For prophylactic applications, the pharmaceutical compositions of the present invention are administered to a host susceptible to the Aβ-related disease, but not already suffering from such disease. Such hosts may be identified by genetic screening and clinical analysis, as described in the medical literature (Goate, 1991). The pharmaceutical compositions will be able to inhibit or prevent deposition of the Aβ plaque at a symptomatically early stage, preferably preventing even the initial stages of the β-amyloid disease. The amount of the compound required for such

prophylactic treatment, referred to as a prophylactically-effective dosage, is generally the same as described above for therapeutic treatment.

The compositions of the present invention may be advantageously packaged into a kit comprising the active reagent(s) a suitable container means and even instructions for use of said kit. The reagent(s) of the kit can be provided as a liquid solution, attached to a solid support or as a dried powder. Preferably, when the reagent is provided in a liquid solution, the liquid solution is an aqueous solution. Preferably, when the reagent provided is attached to a solid support, the solid support can be chromatograph media, a test plate having a plurality of wells, or a microscope slide. When the reagent provided is a dry powder, the powder can be reconstituted by the addition of a suitable solvent, that may be provided.

The container means will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the reagents may be placed, and preferably suitably aliquoted. Where a second reagent is provided, the kit will also generally contain a second vial or other container into which this additional reagent may be placed. The kits of the present invention will also typically include a means for containing the reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

K. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

A parallel β-strand domain of Alzheimer's Aβ fibrils determined using solid state NMR spectroscopy

The present example describes the uses of DRAWS ^{13}C solid state nuclear magnetic resonance (NMR) studies (Gregory *et al.*, 1997) to measure precise distances between individual Aβ peptides in the plaques of the Aβ peptide that are characteristic of AD. Using synthetic peptides containing a single ^{13}C incorporated at different positions, the inventors have found that the arrangement of adjacent peptides is highly specific, consistent with a model of parallel, but not anti-parallel β-strands aligned precisely at the positions of Glutamine-15 and Lysine-16. These studies demonstrate, for the first time, a generalizable technique by which solid state NMR may be used to determine the three-dimensional structure of Aβ in an amyloid plaque.

A. Methods

a. Peptide Synthesis

Peptide synthesis and sample preparation: 1-^{13}C-L-lysine and 1-^{13}C-L-glutamine were obtained from Cambridge Isotope Laboratories. Fmoc and Tboc protection was performed by Midwest Biotech, Inc. Peptides were synthesized using standard Fmoc chemistry on an ABI model 43IA peptide synthesizer. Peptides were purified by multiple ether extractions or by HPLC and verified by MALDI-TOF mass spectroscopy and reverse phase RPLC to be >99% pure. tTg (Sigma) crosslinking of the peptides was performed as described by Ikura *et al.* (1993); the crosslinked products were analyzed by Tris-Tricine polyacrylamide gel electrophoresis. For fibrilization, peptides were solubilized in water, the pH of which was approximately 3.0 due to residual trifiuoroacetic acid in the lyophilized powder; the pH was gradually adjusted to 7.4, and the samples were gently agitated at room temperature for 2-3 days under nitrogen. Under these conditions, Lee *et al.* found the 10-35 peptide to add to plaques isolated from Alzheimer's patients' brains (1995). Fibril formation was monitored by the disappearance of the monomer peak by HPLC and confirmed by electron microscopy. Once fibril formation was complete, the

samples were either lyophilized and stored at -20°C or flash-frozen and immediately inserted into a NMR tube pre-chilled to -80°C.

The peptides generated include those given in Table 5.

Table 5: Peptides synthesized. The Sequence is given in SEQ ID NO:2

Peptide Number	Peptide Sequence
Peptide 1	^{10}YEVHH^{15}Q***K**LVF^{20}FAEDV^{25}GSNKG^{30}AIIGL^{35}M
Peptide 2	^{10}YEVHH^{15*}**Q**KLVF^{20}FAEDV^{25}GSNKG^{30}AIIGL^{35}M
Peptide 3	^{10}YEVHH^{15*}**Q**K*LVF^{20}FAEDV^{25}GSNKG^{30}AIIGL^{35}M
Peptide 4	^{10}YEVHH^{15}QK***L**VF^{20}FAEDV^{25}GSNKG^{30}AIIGL^{35}M
Peptide 5	^{10}YE***V**HH^{15}QKLVF^{20*}**F**AEDV^{25}GSNKG^{30}AIIGL^{35}M
Peptide 6	^{10}YE***V**HH^{15}QKLVF^{20}FAEDV^{25}GSNKG^{30}AIIGL^{35}M
Peptide 7	^{10}YEVHH^{15}QKLYF^{20*}**F**AEDV^{25}GSNKG^{30}AIIGL^{35}M
Peptide 8	^{10}YEVHH^{15}QKL***V*****F**^{20}FAEDV^{25}GSNKG^{30}AIIGL^{35}M
Peptide 9	^{10}YEVHH^{15}QKL***V*****F**^{20}**F**AEDV^{25}GSNKG^{30}AIIGL^{35}M

In the solid state NMR data presented herein, the distance between two interstrand *Lys16 residues was found to be 5 ± 0.5 Å. In contrast, the two *Q15 were found to be ≈7 Å apart. The inventors synthesized two additional peptides 3 and 4 to test of the antiparallel arrangement. In Peptide 3, the interstrand distance between Glnl5 and Leu17 should be ≈5.0 Å in the antiparallel β strand conformation, *i.e.* similar to the interstrand distance between Lys16 residues. Peptide 4 is a negative control, since the interstrand distances between Leul7 would be too far apart in an antiparallel β sheet to show coupling. Coupling would be seen in a parallel β conformation.

The model of antiparallel β strands would then be further propagated using peptides 5, 6 and 7. In Peptide 5, propagation of the antiparallel β strands would give interstrand distances of ≈ 5 Å between Vall2 and Phe20, but no signal for intrastrand labels would be detectable. Peptides 6 and 7 are, in essence, controls: there should be

a signal related to the single ^{13}C label in each strand, but no coupling would be observed.

An additional set of studies will use multiple (triple) quantum filters. In conclusions herein, it is observed that the Lys16 and Gln15 residues in the oligomer were equivalent. If this is so, the signals from a double labeled peptide containing ^{13}C-Lysl6 and ^{13}C-Glnl5 would be removed by a triple quantum filter. This peptide has already been synthesized, and the triple quantum filter study is in progress. The particular peptides made to tested this are peptides 8 and 9.

Peptide 8 serves as a secondary structure-sensitive distance control. In the β strand conformation, the intrastrand distance will be fixed by the secondary structure. In contrast, interstrand contacts will not result in coupling that can pass through a triple quantum filter. Peptide 9 is a positive control for the triple quantum filter studies; the distances between labels is < 5 Å, and the signal should be able to pass through a triple quantum filter.

As controls to help calibrate the distance measurements in the triple quantum filter studies, the inventors synthesize the two following tripeptides:

Peptide 10 $^{*}\mathbf{A}^{*}\mathbf{G}\mathbf{G}$

Peptide 11 $^{*}\mathbf{A}^{*}\mathbf{G}^{*}\mathbf{G}$

The only difference between Peptide 10 and 11 is that in Peptide 10, only the first two residues are labeled, and in Peptide 11, all three are labeled. Since the dimensions of this peptide are known with great precision from a crystal structure, the inventors will use this peptide to calibrate distances in the triple quantum filter: the signal should pass through the triple quantum filter for Peptide 11, but not Peptide 10. The distance obtained from this measurement will be compared to both the crystal structure and that obtained from a quantum mechanical treatment.

b. Solid state NMR:

Magic angle spinning (MAS) solid state NMR studies were performed on a Bruker DMX NMR turned to ^{13}C frequency using the DRAWS (Gregory *et al.*, 1997) and DQDRAWS (Gregory *et al.*, 1996) pulse sequences. Samples were spun at 4525 (±3) Hz. The ^{13}C RF power level was set to 60 kHz. The ^{1}H decoupling level was 120 kHz. Spectra from DRAWS and DQDRAWS studies were processed and analyzed as previously described (Gregory *et al.*, 1996; 1997). Simulations were performed using CHARM as described by Gregory *et al.*, 1997. Models of parallel and antiparallel β-strands, in sheets and barrels, were drawn from crystal structures in the Brookhaven Protein Data Bank; parallel and anti-parallel β-strand distances from the crystal structures were compared to experimental data using the Insight II software package (MSI).

c. Peptide purification and analysis:

For many peptides, first the scavengers and other low molecular weight contaminant are removed by a preliminary gel filtration chromatography step using Sephadex G-25. This is followed by preparative reverse phase HPLC. In the case of βA peptides, however, the inventors have modified this procedure because of unacceptable losses in the first step. In addition, the inventors observed that adequate removal of scavengers was achieved by repeated extractions using diethyl ether and/or ethyl acetate. Peptides are dissolved in 0.1% (v/v) TFA in water and injected onto C4 or C18 preparative reverse phase HPLC columns, typically from Rainin or Dynamax. As described above, a dramatic improvement in yields from chromatography is achieved by heating the columns to 70°C; this temperature is associated with a conformational changed that can be observed by CD spectroscopy. The inventors have obtained peptide that is >98% pure by analytical reverse phase HPLC, amino acid sequencing, electron spray mass spectrometry, amino acid sequencing, and analytical Tris-Tricine SDS-PAGE using this procedure.

B. Results and Discussion

It has been shown, by electron microscopy and x-ray scattering, that Alzheimer's amyloid plaques consist of a highly ordered, fibrillar structure which can be replicated *in vitro* using synthetic peptides. The inventors have developed a generalizable approach, using solid state NMR and specifically labeled synthetic peptides, by which determination of the specific structure of the peptide in its plaque-conformation is possible.

In recent years, solid state NMR has often been looked to as the "new solution" to solving structures of insoluble proteins, such as amyloids, or proteins difficult to crystallize, such as membrane proteins. Additionally, solid state NMR studies can determine distances to within 0.2 Å (Gregory *et al.*, 1997), more precise than the current resolution of any other technique. However, specific structures of complex molecules require the incorporation of specific labels (^{13}C or ^{15}N) at distinct positions. The decision of where to place these labels, in the context of a large molecule, is non-trivial. Thus, to date, these studies have basically been limited to small peptides or oligonucleotide sequences, usually those with known crystallographic structures (Gregory *et al.*, 1997). In 1996, Lansbury *et al.*, published solid state NMR data on a 9-amino acid version of the Aβ peptide, measuring intramolecular distances. However, this work was complicated by additional intermolecular contacts and, due to the short length of peptide studied, could not be directly extrapolated to the general Alzheimer plaque itself.

For the inventors' studies, a 26-amino acid peptide, comprising residues 10-35 of the Aβ peptide (SEQ ID NO:2). This peptide was chosen because it is more soluble, than the native peptide, yet still retains the ability to add to authentic plaques isolated from AD patients (Lee *et al.*, 1995). Furthermore, like the native peptide (Dudek and Johnson, 1994), the inventors found that this peptide could be specifically cross linked by the enzyme tissue tranglutaminase (tTg) at the positions of Lys- 15 and Gln-16 of adjacent peptides.

Analysis of the crosslinked peptide products, when analyzed by Tris-Tricine SDS-PAGE gel (FIG. 1) revealed that the reaction appears to be not only specific to these residues in the sequence, as found by Ikura *et al.* (1993), but also specific to the structure of the small amyloid aggregates, as only products up to the size of a hexameric complex are observed. This led the inventors to hypothesize that the Gln-15 of one peptide strand might be located near the Lys-16 of an adjacent strand in the amyloid plaque, hence suggesting initial sites for the placement of ^{13}C labels for solid state NMR studies. Additionally, as these positions are adjacent in the sequence of the peptide, molecular modeling of β-strands indicated that the inventors would be able to distinguish between parallel and anti-parallel strands using peptides incorporating only a single label (FIG. 2A and FIG. 2B), thus eliminating the complication of intra-strand contacts in the NMR studies. That is, in one set of studies the inventors could study fibrils made up of peptides containing only a single ^{13}C at Gln-15 and in a separate set of studies the inventors could examine fibrils containing only a single ^{13}C at Lys-16. In compilations of possible parallel and antiparallel β-strand distance measurements drawn from crystal structures in the Brookhaven Protein Data Base, of the more than 20 possible parallel and anti-parallel arrangements examined only the parallel arrangement fit the NMR data.

The initial studies employed a pulse sequence containing double quantum filter (DQDRAWS) (Gregory, 1996) to determine whether either the 1-^{13}C-Q or 1-^{13}C-K containing peptides had any measurable interstrand contacts. Briefly, the DRAWS pulse sequence (Dipolar Recoupling in a Windowless Sequence) measures dipolar (non-covalent) interactions of ^{13}C atoms. The double quantum filter serves to remove any single quantum (non-coupled) signals from the study. All of the inventors' experimental samples contained a small amount of unlabeled hexamethyl benzene (HMB) as an internal control. The only possible signals from the HMB are due to natural abundance ^{13}C; the likelihood of two ^{13}C's to naturally occur next to each other is very small (0.01%); thus signal from HMB may be considered to be only single quantum and should not pass through the double quantum filter. Likewise, in labeled samples, only two ^{13}C atoms within 6 Å will be detectable in the DQDRAWS

study. The inventors found that those peptides containing 1-^{13}C at the position of Gln-15 and those containing 1-^{13}C-Lys-16 both had detectable DQ signal (FIG. 3A and FIG. 3B). Compared to control samples, this signal strength placed the inter-peptide distance between 4 and 6 Å (FIG. 3C).

The next set of studies took the same 1-^{13}C-Lys-Aβ and 1-^{13}C-Gln-Aβ samples through a series of DRAWS mixing times, without the DQ filter. In samples with known crystal structures, it has been found that the combination of DRAWS with a "brute force" quantum-mechanical distance simulation enables the precise measurement (within 0.2 Å pairs)(Gregory *et al.*, 1997). As shown in FIG. 4A and FIG. 4B, the inventors found the distance for both the 1-^{13}C-Gln-Aβ and 1-^{13}C-Lys-Aβ samples to be 5.0 Å (±0.3); this was true both for repeated measurements of the same sample and for repeated sample preparations. Interestingly, the fit to a 5.0 Å distance was valid only for simulations in which each spin was coupled to 2 other spins at identical distances apart (FIG. 4A). Models of 2 spins in isolation, at a distance of 4.5 to 5 Å, matched the range of DRAWS data collected, but not the shape of the curve (FIG. 4B). Similarly, models of 3 spins where one pair of contacts is close and the second pair is far, such as might be seen in a linear arrangement of 3 spins, failed to fit the data.

Finally, the inventors employed molecular modeling to analyze possible peptide conformations consistent with these distance constraints. Examination of over 20 possible β-strand configurations (parallel, anti-parallel, sheets, barrels, and different alignments of the two strands), revealed, surprisingly, only one possible solution: multiple parallel β-strands aligned at exactly the position of the carbonyls (1-^{13}C) of the glutamines and lysines at positions 15 and 16 (FIG. 2B). At first, the inventors considered that the data might actually represent some combination of parallel and antiparallel β-strands, or staggered parallel β-strands. However, the simulations for 2 and 3 spins revealed that not only is the arrangement parallel for both labeled positions, but in both cases the interactions must be repeated over and over again. This is consistent with the hypothesis that core of fibrils is repeated

endlessly, perhaps in a circular fashion a predicted by the inventors' enzymatic data (FIG. 1) and modeled in FIG. 5.

EXAMPLE 2

Synthetic PEG Aβ(10-35) Conjugate Forms Soluble β Sheet Fibrils

A. Methods

a. Synthesis of N- and C-PEG-Aβ(10-35)

N-terminal PEG- Aβ(10-35) was synthesized by the manual addition of carboxy-PEG to deprotected Aβ(10-35) still attached to the resin. Coupling was carried out by the repeated addition of carboxy-PEG + 4 equivalents of BOP reagent, until manual qualitative ninhydrin test revealed no remaining free amines. The preparation of C-PEG- Aβ(10-35) was slightly less straightforward, and was carried out as shown in FIG. 6.

b. Circular Dichroic Spectroscopy of PEG-Aβ(10-35)

Circular dichroic (CD) spectra were measured at variable pH using peptide dissolved in a citrate/phosphate buffer system with the salt concentration held constant at 200mM. Data were collected on a Jasco J-600 spectropolarimeter at room temperature using either a 0.01 or 0.1 cm pathlength cuvette. The concentration of peptide by dry weight was 1.74 mM. For studying the effect of peptide concentration on CD spectra, peptide was dissolved into a concentrated stock at 3.8 mM, and CD spectra measured of the stock and various dilutions made with phosphate/citrate buffer at pH 5.8 to achieve peptide concentrations from 0.022 to 3.8 mM.

Circular dichroic (CD) spectra show $[\theta]_{217}$ as functions of peptide concentration. For the concentration dependency, one can derive the equation:

$$T = n - 1\sqrt{\frac{K_d\left(1 - \frac{(\theta_{ex} - \theta_A)}{(\theta_M - \theta_A)}\right)}{n\left(\frac{(\theta_{ex} - \theta_A)}{(\theta_M - \theta_A)}\right)^n}}$$

where $[\theta]_{ex}$, $[\theta]_A$, and $[\theta]_M$ are mean residue ellipticities of the experimental sample, the monomer and the aggregate respectively at 217 nm, T is the total analytical peptide concentration, K_d is the dissociation constant, and n is the number of peptide molecules per aggregate. For the pH dependency, one can derive the equation:

$$\theta_{217} = \frac{\alpha T}{\left(1 + \frac{H}{K_1} + \frac{K_2}{H}\right)}$$

where K_1 and K_2 are the constants for the two relevant dissociations.

c. NMR Spectroscopy:

At concentrations between 1 and 5 mM, lyophilized peptide was dissolved in 10% D_2O/H_2O and the pH was adjusted with NaOH/HCl, and the final volume adjusted to 600 µl. The samples were then centrifuged for 15 min at 14,000 X g. ^1H NMR data were collected on either a Varian 500 or 600 MHz NMR spectrometer with a 5mm probe using pulse field gradient solvent suppression. The solvent and polymer signal were saturated at a frequency which gave the best signal suppression of both peaks. Typical 2D ^1H NMR spectra were collected with 220 to 256 FIDs of 2 to 4K data points, 32 to 128 scans per HD, and spectral width of 5000 or 6000 Hz. Standard Varian pulse sequences, data processing and data acquisition were used. The chemical shifts were referenced to an internal DSS standard set to 0.0 ppm. All data was acquired at 25°C or 10°C.

d. Electron Microscopy

Samples were prepared by dissolving dry peptide to concentrations in the mM range in citrate/phosphate buffer containing 0.1% (w/v) NAN_3. Then the samples were centrifuged for 15 min at 14,000 X g and incubated for four days at room temperature. For EM, samples were applied to a glow discharge 400 mesh carbon-coated support film, followed by staining with 1% uranyl acetate. Micrographs were recorded using Philips EM 300 at magnifications of 45,000.

e. Separation of PEG-derivatized from non-derivativatized peptides.

To separate PEG-derivatized from non-derivativatized peptides, the inventors employ a capping procedure. Free amino groups are biotinylated at pH 7 to target amino groups. Nonderivatized peptides are then be removed using immobilized streptavidin which binds the biotin groups. Although it is possible that a small amount of NH2 groups from lysine side chains will also be coupled to biotin, these will be removed by the same reaction with streptavidin. An additional procedure for separating PEG peptides from underivatized peptides and unreacted PEG is equilibrium density gradient ultracentrifugation, as these species are of distinct and widely disparate densities.

B. Results and Discussion

NOESY and TOCSY spectra obtained give nearly identical results to those obtained by Lee *et al.* (1995) for Aβ (10-35). N-PEG- Aβ (10-35) stayed in solution indefinitely at pH 3.2, but slowly became insoluble at pH near neutrality (pH 6.8). The major difference is the concentration of PEG- Aβ (10-35) that can be reached, resulting in a much higher quality spectra of the PEG- Aβ compared to non-derivatized Aβ. Table 6 shown below is a chart comparing the conditions used by the inventors to those of Lee *et al.* (1995).

Table 6 Comparison between Inventors data with that of Lee *et al.* 1995

COMPARISON OF NMR CONDITIONS		
	Lee *et al.*	**The Present Invention**
NMR	750 mHZ	500 mHz
Probe	10mm Pulse Field Gradient Probe	5mm Probe
Water Suppression	Wave-Form Gradient Suppression	Presat
Temperature	10°C	20°C
Sample Conc.	0.25-0.5 mM	5 mM
Sample Volume	3.5 ml	0.5 ml
Data Acquisition	8K by 512	4K by 256
Experiment Time	Days	12 h

The data presented herein demonstrate that the ability of PEG- Aβs to remain in solution. The improved solubility may be related to steric hindrance in either the aggregation of small oligomers into polymers, or the growth (one molecule at a time) of oligomers into fibrils. In either case, the PEG- Aβs may be stuck in the stage of small oligomers.

Circular dichroic spectroscopy showed that N-PEG- Aβ(10-35) has a spectrum quite similar to that of non-derivatized Aβ(1-39), and undergoes a similar pH dependent shift in the spectrum: (FIG. 9B). This shows the feasibility of using N-PEG-Aβ(10-35) as a homologue of the non-derivatized peptide.

FIG. 7B shows the CD spectra showing $[\theta]_{217}$ as functions of pH concentration and FIG. 7C shows CD spectra show $[\theta]_{217}$ as functions of peptide concentration. From pH 3-7, **1** gave clear solutions while the native peptide under similar conditions of pH, ionic strength and concentrations formed gels or precipitates. FIG. 7A shows a dramatic pH induced transition from random coil to a β sheet. There was a modal increase in β content (decreasing $[\theta]_{217}$ from pH 2.7 to 4.5, followed by the apparent loss of β content due to precipitation of peptide from pH 4.5 to 7.6 (FIG. 7B). From the pH dependency curve, the inventors calculated two pKs, 3.94 and 6.96, indicating interactions between E11 and H13 in a parallel β sheet, stabilizing the transition to β sheet and formation of aggregates at higher pH. There was an equally marked concentration induced transition (FIG. 7C), analysis of which was consistent with a reversible monomer-oligomer equilibrium; curve-fitting indicates a hexameric or heptameric species.

Solution 2-D ^{1}H NMR studies (NOESY and TOCSY, 8,9) showed a pH dependent transition from an unstructured peptide to one with a signature consistent with extended conformation, as was also seen by Lee *et al.* (1995) for nonmodified Aβ(10-35). Although the extended conformation is similar to the β strand or sheet conformation in that the peptide chain is elongated, there is a notable absence of the

downfield shift of Cα proton resonances diagnostic of β strand conformation (Dudek and Johnson, 1994). CD spectra of the samples used for the NMR studies (*i.e.* the latter at lower ionic strength than the former) were virtually identical to the curve shown in FIG. 7A for 0.6 mM peptide in a buffered solution. When the pH was raised slightly (to ≈5.8) or the peptide concentration is increased, there was little change in the 1D spectrum, but there was a loss of signal and increased line broadness. Line broadening is consistent with the formation of aggregates. The inventors infer that any β sheet present in the latter NMR samples, are invisible to the NMR due to their large sizes and/or lower abundances of any smaller aggregates. At pH 3.0, the peptide was unstructured, again indicating a transition from so-called "random coil" to an extended structure.

Solution NMR data and light scattering analyses were also consistent with the formation of large MW species under similar conditions. However, in contrast to the nonmodified peptide, these associations are completely reversible, and occur at nearly two order of magnitude higher concentration, and at pH closer to neutrality, and may allow the obtaining of high quality 2-D NMR spectra, *e.g.*, at concentrations of 1 mM peptide, pH 5.8.

Electron micrographs prepared from **1** at pH 3.0 were virtually indistinguishable from those of nonmodified Aβ 10-35. Indeed, this is somewhat surprising in that the PEG moiety might be expected to increase the fibril diameter. Instead, the fibrils of PEG Aβ appeared as if negatively stained, and were no thicker than fibrils of nonmodified Aβ, consistent with the notion that the PEG coats exterior of the fibril. At pH 5.8 or 7.4, however, the nonmodified peptide fibrils aggregated laterally into bundles, whereas **1** remained as linear, non-aggregated fibrillar strands, and only slowly (3 days) formed occasional lateral aggregates at pH 7.4. Bundles of **1** were never observed at pH 5.8.

EXAMPLE 3

Structure of the $A\beta_{(10-35)}$ Fibril

The present example yields a high resolution description of fibers formed from $A\beta_{(10-35)}$ peptides. Aβ fibers were studied both in solution and in fibrillar arrays. The structure consists of four to six laminated β-helices propagating along the helical axis. Each peptide strand is oriented perpendicular to the helical axis in a parallel β structure, with each amino acid residue in register between the strands.

A. Methods

a. Peptide Synthesis

Aβ-PEG containing either 1-^{13}C-Leu or 1-^{13}C-Val was synthesized using standard fluorenyl-methoxy carbonyl protocols on PAP Tenta-Gel (Rapp Polymere) to introduce the label at position 17 or 24 (Burkoth *et al.*, 1998). Cleavage by TFA and deprotection yielded a linear PEG 3000 covalently bound to the carboxyl terminus of the peptide. Peptide purity, determined by synthetic coupling yields and reverse phase HPLC of CNBr cleavage products, was >96%. Molecular masses of all peptides were verified by MALDI-TOF mass spectroscopy.

b. Solid State NMR

Aβ-PEG fibrils were prepared as described in Benzinger *et al.* (1998) and CP/MAS ^{13}C-NMR studies were performed on a Bruker Avance DSX spectrometer at 50.3 MHz using the DRAWS sequence (Benzinger *et al.*, 1998; Gregory *et al.*, 1997). Spectra were acquired on 10 mg samples with >500 scans for the fibril samples and >1800 scans for the non-fibrilized control sample of 1-^{13}C-Leu_{17}-Aβ-PEG or 1-^{13}C-Val_{24}-Aβ-PEG. Hexamethylbenzene (8-12 mg) served as internal control. Samples were spun at 4525 (±3) Hz, the ^{13}C RF power level was 38.5 kHz, the ^{1}H decoupling level was 120 kHz, and the spectra were processed and analyzed as described in Gregory *et al.* (1997). Virtually all β-sheet carbonyl chemical shifts are superimposable at δ 171 ppm in the solid state NMR study and the signal attributable to the unlabelled amino acids was subtracted from the total signal.

Simulated data were created by numerical calculation using a density matrix approach (Gregory *et al.*, 1997). The input parameters to the numerical calculation program included the chemical shift tensor (CSA) elements for the spin-1/2 nuclei, the dipolar coupling strengths, Euler angles which rotate the CSA tensors from the molecular frame to their respective principle axis systems, an initial density matrix p(0), an observable, and any relevant relaxation parameters. In the present study, the Euler angles were set to zero, as it was determined that they had a negligible effect on the simulated curves. The CSA parameters were taken from Ye *et al.*(Ye *et al.*, 1993). The time increment was typically 2-3ms. Relaxation effects were modeled by multiplying the single quantum density matrix elements by an exponential factor at the end of each time increment. Data from the non-fibrilized sample was used to determine the appropriate single quantum relaxation constant. The program also performs a powder average of 2000 randomly selected crystallite orientations.

c. Electron Microscopy

Samples of $A\beta_{(10-35)}$/Aβ-PEG were prepared for EM by dissolving dry peptide to 2 mM in water containing 0.1% NaN_3 (pH ~ 3.0). The samples were mixed, centrifuged for 15 min at 14,000 X g and incubated for 1 h. The mixtures were then diluted to 1 mM in phosphate buffer at pH 5.7 and allowed to stand at room temp for 3 days. Samples were applied to a glow discharge 400 mesh carbon coated support film, followed by staining with 1% uranyl acetate. Micrographs were recorded using Philips EM 300 at magnifications of 100,000, 45,000, and 10,000.

d. Small Angle Neutron Scattering

SANS studies were performed at the time-of-flight small-angle neutron diffractometer (SAND) at the Intense Pulsed Neutron Source of Argonne National Laboratory (Crawford and Thiyagarajan, 1997). Pulsed neutrons, λ 0.5 -14 Å, were detected at a fixed sample-to-detector distance of 2.0 m with a 128×28 array of position sensitive gas filled 40×40 cm^2 proportional counters, and the wavelengths were measured with time-of-flight by binning the pulse to 68 constant $\Delta\lambda/\lambda = 0.05$

time channels. This instrument provides a useful range of momentum transfer ($Q = 4\pi\sin(\theta)/\lambda$, where θ is half the scattering angle and λ is the wavelength of the probing neutrons) of 0.0035 - 0.6 $Å^{-1}$ in a single measurement. The measurements on the samples in 12% D_2O were made on SAD (Thiyagarajan *et al.*, 1997) which is similar to SAND, but the Q range is limited to 0.005 – 0.25 $Å^{-1}$.

$A\beta_{(10-35)}$ (5.77 mg/ml) and Aβ-PEG (11.5 mg/ml) in 99 % D_2O buffer at pH = 5.7 were analyzed in 5 mm quartz cells and $A\beta_{(10-35)}$ (6.1 mg/ml) and Aβ-PEG (19.5 mg/ml) in 12 % D_2O buffer at pH = 5.7 were analyzed in 1 mm quartz cells. The evolution of the scattering signal from the samples in 99% D_2O was followed for several h at 22 °C, with each measurement lasting for 1 hr, to ensure that the samples had reached thermodynamic equilibrium at the length scales being measured. Each of the samples in 12% D_2O (low contrast for protein and almost no contrast for PEG) were measured using SAD for about 20 h. The scattering data were corrected for the background from the instrument, the sample cell, and the solvent and the differential scattering cross-section was placed on an absolute scale (Thiyagarajan *et al.*, 1997) in the units of cm^{-1} and the data were further normalized for the concentration of sample in mg/cm^3.

The differential scattering cross-section, I(Q), in the absence of interparticle correlation, is given by

$$I(Q) = N_p \cdot V_p^2 \cdot (\rho_p - \rho_r)^2 F(Q)$$

where N_p is the number of particles in unit volume, V_p is the volume of the particles, F(Q) is the form factor describing the shape of the particles, ρ_p and ρ_r are the neutron scattering length density of the particle and the solvent, respectively. The scattering length density ρ for any system can be calculated by using

$$\rho = N_A d \sum \frac{b}{M}$$

where b and M are the neutron scattering length and mass of individual atoms respectively, d is the density of the system and N_A is Avogadro's number.

The difference in the scattering length densities of the particle and the matrix is known as the contrast. I(Q) will be zero if the scattering length density of the particle is equal to that of the solvent. Since the scattering length density of H_2O, D_2O, PEG, and the peptide are -0.56×10^{10} cm^{-2}, 6.34×10^{10} cm^{-2}, 0.57×10^{10} cm^{-2} and 1.93×10^{10} cm^{-2}, respectively, the coherent scattering from PEG can be eliminated if 16% D_2O buffer is used (Thiyagarajan *et al.*, 1995). For the present contrast matching study, however, 12 % D_2O buffer was used as a compromise to reduce the PEG scattering to an insignificant level, but still have sufficient contrast for the scattering from the peptide.

Absolute SANS data allow for the evaluation of mass-per-unit length for the rod-like particles from the y-intercept value of the modified Guinier plot (Ln[Q.I(Q). vs. Q^2 plot] by using

$$M_L = \frac{1000 I_c(0) d^2 N_A}{\pi c (\rho_p - \rho_s)^2}$$

Here $I_c(0)$ is the exponential of the y-intercept from the modified Guinier plot, c is the concentration of the sample in mg/ml determined by amino acid analysis, d is the density of the peptide (inverse of partial specific volume), ρ_p and ρ_s are the neutron scattering length density values of peptide and solvent respectively and N_A is the Avogadro's number. A partial specific volume of 0.744 cm^3/g and ρ_p of $1.82 \times 10^{10} cm^{-2}$ for the peptide, determined from its atomic composition and the neutron scattering cross-sections of elements, was used.

B. Results and Discussion

The inventors define, using a combination of synthetic model peptides, solid-state NMR, and small angle neutron scattering (SANS), the three dimensional structure of the $A\beta_{(10-35)}$ fibril. The amino acid sequence of Aβ can be separated into distinct domains: a hydrophilic N-terminus (aa 1-16), a central hydrophobic region (aa 17-21), and a long hydrophobic C-terminus (aa 29-43). $A\beta_{(10-35)}$, a peptide comprising the core residues 10-35, preserves these characteristic domains (FIG. 8A) (Esler *et al.*, 1996) and can be used to prepare homogeneous fibrillar arrays. Using CP/MAS solid state NMR, it has been demonstrated that $A\beta_{(10-35)}$ exists as a single repeating parallel β-strand with amino acid residues in register within the fibril (Benzinger *et al.*, 1998). These studies employed the DRAWS pulse sequence (Gregory *et al.*, 1997) to measure inter-strand distances *via* the incorporation of ^{13}C-isotopic labels at key positions across the entire sequence of the peptide. Precise distance measurements at the backbone carbonyls of residues 12, 15-18, 20, 24, 25, 26, 29, 33, and 34 established that a repeating array and a common orientation of parallel β-strands exists with each amino acid residue in register with like residues on both sides within the fibril (FIG. 8B).

This parallel β-sheet orientation clusters the hydrophobic C-termini of each peptide and predicts the propagation of a hydrophobic face along the entire length of the fibril. *A priori*, such a solvent exposed hydrophobic surface would be expected to dominate the behavior of the fibrils. To investigate this possibility, a polyethylene glycol block was synthetically attached at the C-terminus of $A\beta_{(10-35)}$, Aβ-PEG, as shown in FIG. 8A (Burkoth *et al.*, 1998). This modification greatly improved solubility without removing the ability of the peptide to form fibrils. Most importantly and unlike the native peptide, the formation of fibrils by Aβ-PEG was completely reversible, allowing for the definition of the thermodynamics of fibrillogenesis. CD analyses of Aβ-PEG demonstrated a concentration dependent equilibrium in which the unstructured peptide self-associated into a β-strand oligomer, most consistent with a hexamer, during fibrillogenesis (Burkoth *et al.*, 1998). Unlike

the native peptide, Aβ-PEG fibrils rarely laterally associated into tangled webs and instead had the appearance of "combed hair" by EM. It was concluded that the PEG chimera prevented lateral association of the fibrils and thereby inhibited the irreversible step in fibrilogenesis (Burkoth *et al.*, 1998).

The more complex models of Aβ fibril formation, specifically those which place the hydrophobic C-termini in the core of the fibrils (Fraser *et al.*, 1994; Lansbury *et al.*, 1995; Malinchik *et al.*, 1998; Lazo and Downing 1998), should be completely disrupted by PEG attachment. Several approaches were taken to compare the fibrils formed by Aβ-PEG with those of $A\beta_{(10-35)}$. First, Aβ-PEG containing either 1-^{13}C-Leu_{17} or 1-^{13}C-Val_{24} isotopic label was prepared. In fibrilized samples, a strong dipolar coupling existed between the labels in each peptide as detected by solid-state NMR DRAWS studies. Contact distances, modeled as a repeating array, of 5.2 (±0.3) Å for 1-^{13}C-Leu_{17} and 4.8 (±0.3) Å for 1-^{13}C-Val_{24} were determined as shown in FIG. 8C. The contacts were fibril-dependent, as non-fibrilized peptide amorphous precipitates had no measurable Leu_{17}-Leu_{17} or Val_{24}-Val_{24} inter-peptide dipolar coupling interactions. Analysis of structures in the Brookhaven Protein Data Bank had previously established that only β-structures with residues in exact register could accommodate this carbonyl-carbonyl contact distance between strands (Benzinger *et al.*, 1998), confirming the basic inter-peptide alignment of Aβ-PEG as identical to that of $A\beta_{(10-35)}$.

Secondly, EM was used to examine the morphology of fibrils formed from mixtures of the two synthetic peptides (FIG. 9). The relative ratio of $A\beta_{(10-35)}$ to Aβ-PEG was adjusted while total peptide concentration remained constant. Fibrils composed entirely of $A\beta_{(10-35)}$ contained primarily twisted, paired fibrils, ~90 by ~160 Å across the narrowest and thickest dimensions, with a superhelical repeat distance of ~1100 Å, and occasional monomeric fibrils ~80 Å diameter (FIG. 9A). As the Aβ-PEG component was increased, there was less and less lateral self-association of the fibrils (FIG. 9B, FIG. 9C, FIG. 9D and FIG. 9E). In addition, the EM staining

of the fibrils became more diffuse and a halo around the fibrils became visible. Aβ-PEG fibrils (FIG. 9E) were entirely monomeric with poor edge contrast and diameters >80 Å. These observations are consistent with Aβ-PEG and $A\beta_{(10-35)}$ forming hetero-duplexes with PEG coating the surface of the Aβ fibril to prevent fibril-fibril association (Burkoth *et al.*, 1998). By extension, the hydrophobic C-terminus of $A\beta_{(10-35)}$ would also be localized to the edge of a monomeric fibril and thus exposed to solvent in the absence of pairing. For this reason, in the absence of PEG, non-paired single filaments would be expected to associate so as to sequester the hydrophobic edge away from solvent.

Finally, the radii in solution of both the Aβ-PEG and $A\beta_{(10-35)}$ fibrils were examined by small angle neutron scattering. The SANS data for the samples were interpreted using a modified-Guinier analysis for a rod which involves plotting $\ln[Q \cdot I(Q)]$ versus Q^2 (Thiyagarajan *et al.*, 1995). Rod-like particles give rise to a linear region in the modified Guinier plot in the low Q region ($Q \cdot R < 1.4$) where the radius of the rod R can be derived from the slope of the straight line by the relation $R^2 = -4 \cdot \text{slope}$ (Porod, 1982). If the data are available on an absolute scale, the mass-per-unit length can be determined using the y-intercept of the above fit (Lansbury *et al.*, 1995). FIG. 10A shows that the average radius of $A\beta_{(10-35)}$ in 99% D_2O buffer at pH 5.6 was 46 (±5) Å. In addition, the slightly larger slope in the low Q region is consistent with the presence of rods of a larger radius, 70 (±10) Å, similar to the fibril-fibril association observed by EM in FIG. 9A. The SANS data for Aβ-PEG fibrils under the same conditions yielded a single species with a radius of 65 (±2) Å. Thus, the PEG moiety increases the radius of the fiber by about 20 Å.

Contrast matching, by adjusting the deuteration level of the solvent eliminated the scattering due to PEG and thus selectively measured the peptide portion of the molecule (Thiyagarajan *et al.*, 1995). In 12% D_2O, the contrast factor to render PEG invisible, the radii for Aβ-PEG and $A\beta_{(10-35)}$ fibrils were 47 and 46 (±5) Å, respectively (FIG. 10B). This dramatic loss of the difference in radius between these

two samples in 12% D_2O confirms the conclusions from EM and NMR analyses that the PEG block was at the surface of the Aβ-PEG fibril. Most importantly, the macromolecular organization of the peptide portion of both fibrils is identical.

A fully extended $A\beta_{(10-35)}$ peptide would be ~90 Å in length. The stacking of these peptides into an extended β-sheet, propagating along the fiber axis as shown by the solid-state NMR studies, would give an extended β-sheet, or β-helix conformation (FIG. 11A), a structure proposed to be a common feature of all amyloids (Blake and Serpell, 1996). The CD analysis of Aβ-PEG (Burkoth *et al.*, 1998), and the transglutaminase cross-linking studies of Aβ-PEG and $A\beta_{(10-35)}$ (Benzinger *et al.*, 1998; Burkoth *et al.*, 1998) could be explained by six such β-sheets being laminated together under the conditions used for fibril formation. Such a lamination positions the side chains of Gln_{15} and Lys_{16} for crosslinking between sheets and also places the ^{13}C-labelled carbonyls ~10 Å apart, too far for detectable dipolar coupling. Proof of this lamination was obtained from direct measurements of the density of the fibril from the value of $I_c(0)$. The single fibril of Aβ-PEG in 12% D_2O gave a mass-per-unit length of 2360 (±105) Da/Å. The measured value for Aβ-PEG at 11.5 mg/ml in 16% D_2O (the exact contrast match point for PEG) at pH 5.7 was 2,285 (±105) Da/Å. A 2,900 Da peptide arranged every 5 Å in the sheet would predict a value of 2,320 Da/Å with four laminated sheets. The degree of lamination appears to be very dependent on the conditions used for fibril formation, particularly pH, and this value of four is assigned as a lower limit due of the unavoidable precipitation from highly concentrated peptide solutions and reduction in the concentration during the 20 hr of data collection.

The present data is therefore most consistent with a lamination of up to six sheets, positioned 10 Å apart, for the β-helical $A\beta_{(10-35)}$ fibril (FIG. 11). This arrangement would predict a rectangular rod having dimensions of 90 × 70 Å. Such a structure accounts for the repeating 10 Å and 5 Å x-ray reflections commonly detected in many amyloids (Blake and Serpell, 1996), and most remarkably, confirms a

structural model proposed of almost 25 years ago (Cooper, 1974). Cooper based his model on polysaccharide dye binding agents, specifically I_2 and Congo Red, EM analyses (Glenner *et al.*, 1968), and the initial x-ray diffraction results (Gueft, 1972). It was the common staining of these fibers with polysaccharide dyes that led initially to the term "amyloid" (Virchow, 1854), and the analogy with the organized arrays of the cellulose fibers that allowed him to construct an organized array for the amyloid peptides. The general elements of the model shown in FIG. 11D are lifted directly from that originally drawn by Cooper (Cooper, 1974).

With the precise model now available for the $A\beta_{(10-35)}$ fibril, finer structural features can be determined. The ordered twisting, paired fibrils, commonly observed in EM of $A\beta_{(10-35)}$ (FIG. 11A and FIG. 11B), are completely disrupted by covalent attachment of PEG at the C-terminus (FIG. 9). The parallel β-helix structure places the hydrophobic C-terminal amino acids along one edge, extending along the fiber axis. It is this hydrophobic contact that has been attributed to the fibril-fibril association that is disrupted by PEG attachment (Burkoth *et al.*, 1998). In that context, the super helical twist shown in FIG. 11A and FIG. 11B can be assigned as an antiparallel pair of β-helical bundles with dimensions of 180 × 70 Å as shown in FIG. 11C. An average duplex pitch of ~1100 Å, shown in FIG. 11A and FIG. 11B, is made up of peptides within each β-helix spaced at 5 Å intervals (FIG. 11D), and the individual β-strands must be offset by 1.6°/peptide to define the twist of the β-helix. Such a structural design of paired helices of opposite antiparallel orientation, spiraling to protect a hydrophobic interior is one of nature's most common and fundamental designs, most apparent in Watson-Crick DNA duplexes.

Seilheimer *et al.* (Seilheimer *et al.*, 1997) have shown that deletion of C-terminal hydrophobic amino acids of Aβ results in a loss of twisting and pairing of the resulting fibrils, much as was seen with Aβ-PEG. Moreover, extensive mutagenesis of the amino acid sequences of Aβ did not significantly alter the fibrils as observed by EM, FTIR, and x-ray diffraction. The main difference noted in these structures was that the point mutations influenced the kinetics of fibril formation in a pH dependent

manner (Fraser *et al.*, 1992). Other truncated peptides frequently form fibers, but the observed diameters do not always correlate with extended peptide length, a result that may be complicated by differing degrees of lamination (Teplow, 1998). Finally, construction of an all-D isomer of Aβ was found to reverse the handedness of the fiber twist, without altering the diameter or the pitch (Harper *et al.*, 1997). These findings with Aβ and Aβ truncations suggest a robust structure compatible with that proposed for $A\beta_{(10-35)}$.

β-Helices are often composed of homologous repeating sequences, most notably Tyr, Phe, and bulky hydrophobic amino acids (Leu, Ile, Val) (Nesloney and Kelly, 1996). In the $A\beta_{(10-35)}$ structure, the paired adjacent amino acids His_{13}-His_{14}, Leu_{17}-Val_{18}, Phe_{19}-Phe_{20}, and Ile_{31}-Ile_{32} result in repeating aromatic and aliphatic stacks on both sides of every β-sheet. In addition, the central core of hydrophobic residues, aa 17-21, forms a surface that would promote both vertical β-sheet elongation and sheet-sheet stacking. The dramatic pH dependence for fibril formation, clearly demonstrated with Aβ-PEG (Burkoth *et al.*, 1998), is also a function of these side chain interactions. For example, His_{13} and His_{14}, while on opposite faces of a single β-sheet, would directly interact with the respective His_{14} and His_{13} residues of the adjacent, laminated sheets. In this regard, the addition of Zn^{+2} has been shown to increase the rate of fibril formation in Aβ (Bush *et al.*, 1994) and, indeed, coordination of Zn^{+2} by histidines on facing β-sheets within a β-helix has been observed in the crystal structure of carbonic anhydrase Kisker *et al.*, 1996).

Finally, several lines of evidence suggest that the hydrophobic C-terminus would likely drive the formation of parallel β-strands for Aβ. β-Sheets are typically composed of hydrophobic residues, and Choo *et al.* have shown that the tendency to form β-strand fibrils is directly proportional to the degree of hydrophobicity incorporated into model peptides (Choo *et al.*, 1996). For Aβ, a hydrophobic collapse has been observed by surface balance studies and is correlated with the partitioning of hydrophobic probes (Soregan *et al.*, 1994). It is likely that this association accounts

for the 7 nm "micelles" observed by light scattering (Lomakin *et al.*, 1996), the "spherical seeds" found in the early growing fibers by EM (Seilheimer *et al.*, 1997) and the concentration dependent CD of Aβ-PEG (Burkoth *et al.*, 1998). Most critically, a "micelle-like" association of the hydrophobic C-terminus of Aβ peptides would pre-organize the strands in a *parallel arrangement*, dramatically increasing the effective molarity of this conformation for the formation of the β-helix.

Therefore, the amyloid fiber formed with $A\beta_{(10-35)}$ has many of the features of the full length Aβ fibril, and structural features of this fibril maybe generalizable to other amyloid diseases. Moreover, this structure now represents the largest noncrystalline molecular assembly whose structure has been determined, and the methods outlined here provide the first general strategy for the characterization of such structural arrays. The methodology and the structural insight that it provides make it possible to develop more rational strategies to prevent amyloid formation.

EXAMPLE 4

Preclinical Models

In particular embodiments, it is envisioned that the inhibitors of fibrillogenesis generated herein will be used in therapeutic applications. However, prior to use as therapeutics in humans it may well be necessary to conduct efficacy testing in *in vivo* animal models. The present Example provides methods for such testing.

There are numerous animal models for amyloidogenesis for example, U.S. Patent 5,387,742 describes a transgenic mice displaying the amyloid-forming pathology of Alzheimer's disease. In other models an amyloid enhancing factor is administered to an animal in order to enhance amyloidogenesis. The preparation of amyloid enhancing factor has been described previously in Axelrad, M. A. *et al.* (1982).

Thus, animals are obtained in which one group is a control non-amyloid generating animal that remains untreated. The remaining animals, which are amyloid-

forming, are divided into two groups 1) those which receive the therapeutic composition (*i.e.* for example the PEGylated βA) at 50 mg, 40 mg, 20 mg, or 10 mg by, for example, intraperitoneal injection every 12 h and 2) those which receive a buffered placebo by IP injection. The animals are sacrificed at a time determined by the investigator.

At the termination of the studies, the tissues suspected of having amyloid plaques are fixed for histochemical analysis. Following fixation, the tissues were embedded in paraffin, 8-10 micron sections were cut and stained with Congo Red (Puchtler *et al.*, 1983). The histologic sections are then viewed under polarized light to assess by image analysis the percent of tissue occupied by amyloid. Of course other staining methods employing other dyes or antibody detection may also be used.

The viability of animals will also be indicative of the therapeutic potential of the PEGylated amyloidogenic peptide. For example, animals receiving only the placebo buffer should succumbed to illness or death prior to the termination of the study. Of those animals receiving the therapeutic composition its is expected that more animals in this group survive than in the placebo group.

To approximate a more realistic clinical situation, a separate set of studies may be performed in which the PEGylated treatment was begun after amyloid deposition had already begun. To assess the effect of the therapy on the course of amyloid deposition after amyloid was already present, animals in each group are sacrificed on days 7, 10, 14, and 17. The tissue is processed and evaluated as described above. Control animals will continued to deposit amyloid for 14 days, following which the quantity of amyloid began to decrease. In contrast, the treated group of animals should stop deposition of amyloid. This would demonstrate that the therapy is effective at inhibiting ongoing deposition of amyloid.

Thus, PEGylated amyloidogenic compounds that prove effective in such model studies can be administered as therapeutically effective compounds in clinical studies.

EXAMPLE 5

Clinical Trials of the Use of Fibrillogenesis Inhibitors alone or in Combination with Anti-Amyloidogenesis Drugs in Treating Fibrillogenic Disease

This example is concerned with the development of human treatment protocols using the inhibitors of amyloid fibrilliogenesis, such as for example the PEGylated Aβ peptides of the present invention, alone or in combination with other treatments for plaque related diseases. These treatments will be of use in the clinical treatment of various diseases in which amyloidosis and plaque formation play a role, including Alzheimer's disease, Downs syndrome and other disease listed in Table 1 herein above.

The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art in light of the present disclosure. The following information is being presented as a general guideline for use in establishing blocked amyloidogenic peptides alone or in combination with other drugs in clinical trials.

Patients with an amylodogenic disease or at risk of contracting such a disease are chosen for clinical study will typically have failed to respond to at least one course of conventional therapy. Measurable disease is not required. The only criterion is that these patients have or are suspected to have amyloidogenic plaques and are or have undergone fibrillogenesis. Such fibrillogenesis can be defined and monitored as described herein above.

In an exemplary clinical protocol, patients may undergo placement of a catheter, or other suitable delivery device, in a cavity will provide an effective means of

delivering a therapeutic compounds of the present invention and for sampling the individual for the presence of plaque-forming amyloiodgenic peptides. In the same procedure, the PEGylated peptide compound may be administered alone or in combination with other therapeutic drugs that are commonly used in the treatment of Alzheimer's Disease and other amyloidogenic diseases. The administration may be regional, directly into the fibbrillogenic plaque, or in a systemic manner.

The starting dose may be 0.5mg/kg body weight. Three patients may be treated at each dose level in the absence of grade ≥ 3 toxicity. Dose escalation may be done by 100% increments (0.5mg, 1mg, 2mg, 4mg) until drug related grade 2 toxicity is detected. Thereafter dose escalation may proceed by 25% increments. The administered dose may be fractionated equally into two infusions, separated by six h if the combined endotoxin levels determined for the lot of PEGylated peptide and the lot of second drug exceed 5EU/kg for any given patient.

The PEGylated peptide and anti-amyloidogenic drug combination may be administered over a short infusion time or at a steady rate of infusion over a 7 to 21 day period. The PEGylated peptide compound infusion may be administered alone or in combination with the anti-amyloidogenic drug. The infusion given at any dose level will be dependent upon the toxicity achieved after each administration. Hence, if Grade II toxicity was reached after any single infusion, or at a particular period of time for a steady rate infusion, further doses should be withheld or the steady rate infusion stopped unless toxicity improved. Increasing doses of PEGylated peptide alone or in combination with an anti-amyloidogenic drug will be administered to groups of patients until approximately 60% of patients show unacceptable Grade III or IV toxicity in any category. Doses that are 2/3 of this value could be defined as the safe dose.

Physical examination, plaque measurements, and laboratory tests should, of course, be performed before treatment and at intervals of about 3-4 wk later. Laboratory studies should include CBC, differential and platelet count, urinalysis,

SMA-12-100 (liver and renal function tests), coagulation profile, and any other appropriate chemistry studies to determine the extent of disease, or determine the cause of existing symptoms. Also appropriate biological markers in serum should be monitored.

To monitor disease course and evaluate the anti-plaque responses, it is contemplated that the patients should be examined for appropriate plaques and markers of disease every 4 wk, if initially abnormal. When measurable disease is present, plaque size measurements are to be recorded every 4 wk. Appropriate CAT scanning studies should be repeated every 8 wk to evaluate plaque response. An urinalysis may be performed every 4 wk.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by the disappearance of all measurable disease for at least a month. Whereas a partial response may be defined by a 50% or greater reduction of the sum of the products of perpendicular diameters of all evaluable fibrillogenic plaques or at least 1 month with no plaque sites showing enlargement. Similarly, a mixed response may be defined by a reduction of the product of perpendicular diameters of all measurable lesions by 50% or greater with progression in one or more sites.

Example 6

Blocking Amyloid Plaque Formation

In particular embodiments of the present invention, the inhibition of fibrillogenesis is accomplished by blocking an amyloidogenic peptide with polyethylene glycol. The design of Aβ-PEG, exploits the physical and biological characteristics of PEG. PEG is a chemically inert, achiral, linear, homo-polymer of varying molecular weights, that has unique solubility characteristics; unstructured and soluble in both aqueous and organic solvents (Bailey and Koleske, 1967; Liu and Parsons, 1969; Maxfield and Shepherd, 1975). Because of these physical characteristics, PEG has been used in a variety of applications (Inada *et al.*, 1995;

Harris 1992). Covalently attached PEG has been used as a solubilizing agent to expedite organic chemistry reactions (Han and Janda, 1997) and has been used to solubilize enzymes in apolar aprotic organic solvents (Kodera *et al.*, 1994). PEG can increase the lifetime of pharmacologically active proteins by blocking the surface of the bio-molecule from contact with the immune system and with proteases (Abuchowski *et al.*, 1977). In general, PEG is believed to perturb protein/protein interactions by acting as a stearic barrier.

The C-terminal Aβ-PEG block co-polymer is one solution to inhibiting AD fibril formation by perturbing protein-protein interactions and improving aggregate or fibril solubility. It is contemplated that the general features of this strategy can be extended to other specific inhibitor molecules in two general ways. The first is the use of a different chain length PEG moiety or a molecule that has similar characteristics to that of PEG. In this initial work a covalently attached heterogeneous PEG 3000 polymer was used, but a smaller or more homogeneous PEG molecule could be incorporated in a similar manner using solid phase synthetic methods. Moreover, the characteristics of PEG can be replaced with other solubilizing moieties. Candidate solubilizing molecules considered are sugars, sulfate groups, phosphates or polar peptides/proteins. All of these modifications are available through the same solid phase synthesis methods developed for PEG attachment. The second general strategy contemplated is the location of the covalently attached solubilizing and/or blocking agent. The structure of the fibril described earlier shows that the attachment of a solubilizing molecule could also be incorporated at the N-terminus and on residue side-chain with little perturbation to the fibril structure. For example, solubilizing molecules such as sugars, sulfate groups, phosphates or polar peptides/proteins could be incorporated at the N-terminus and on residue side-chain of the fibril, potentially improving fibril solubility with little structural perturbations. This generality will allow for versatility in tissue targeting and serum lifetime.

* * *

All of the compositions and/or apparatus disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Abrahamson *et al.*, *Biochemical Journal*. 268(2):287-94, 1990.

Abrahamson, *Scandinavian Journal of Clinical & Laboratory Investigation* - Supplement., 226:47-56, 1996.

Abuchowski, van Es, Palczuk, Davis, *J. Biol. Chem.*, 252:3578-81, 1977.

Adachi *et al.*, "Aggregation of hemoglobin S modified by bifunctional imidoesters, *Biochim. Biophys. Acta.*, 742:597-606, 1983.

Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, Cold Spring Harbor, New York, 1988.

Ashburn *et al.* "Amyloid Probes based on Congo Red distinguish between fibrils comprising different peptides," Chemistry and Biology 3:351-358, 1996.

Axelrad *et al.*, "Further Characterization of Amyloid Enhancing Factor," *Lab. Invest.*, 47:139-146, 1982.

Baichwal and Sugden, "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," *In*: *Gene Transfer*, Kucherlapati R., ed., New York, Plenum Press, pp. 117-148, 1986.

Bailey Jr., Koleske, *In: Nonionic Surfactants* (Schick, M.J. ed), Marcel Dekker, Inc., New York, pp. 794-821, 1967.

Bax *et al.*, "The entrapment of polyethylene glycol-bound adenosine deaminase (Pegademase) in human carrier erythrocytes," *Biochem. Soc. Trans.*, 24:442S, 1996.

Bayer and Rapp, "Polystyrene-Immobilized PEG Chains: Dynamics and Application in Peptide Synthesis, Immunology, and Chromatography," *In: Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*, J. Milton Harris, ed., Plenum Press, New York, pp. 325-345, 1992.

Benvenga S. Facchiano A, *Journal of Internal Medicine*. 237(5):525-6, 1995.

Benzinger, Gregory, Burkoth, Miller-Auer, Lynn, Botto, Meredith, *Proc. Natl. Acad. Sci. USA*, 95:13407-13412, 1998.

Bezrukov, "Spatial structure formation in protein gels," *Angewandte Chemie*, 18:599-610, 1979 .

Blake and Serpell, *Structure*, 4:989-998, 1996.

Bobek LA. *et al.*, *Biochemical Journal*. 278 (Pt 3):627-35, 1991.

Buchet *et al.*, "Conformations of synthetic | peptides in solid state and in aqueous solution: relation to toxicity in PC12 cells," *Biochim. Biophys. Acta.*, 1315:40-46, 1996.

Burdick *et al.*, "Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs," *J. Biol. Chem.*, 267(1):546-554, 1992.

Burkoth, Benzinger, Jones, Hallenga, Meredith, Lynn, *J. Am. Chem. Soc.*, 120:7655-7656, 1998.

Busciglio *et al.*, "Generation of beta-amyloid in the secretory pathway in neuronal and nonneuronal cells, Proc. *Natl. Acad. Sci. USA*, 90:2092-2096, 1993.

Bush, Pettingell, Multhaup, Paradis, Vonsattel, Gusella, Beyreuther, Masters, Tanzi, *Science*, 265:1464-1467, 1994.

Campbell, *In*: *Monoclonal Antibody Technology, Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 13, Burden & Von Knippenberg (Eds.), Elseview, Amsterdam, pp. 75-83, 1984.

Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 76:425, 1977.

Carrel and Lomas, "Conformational Diseases," *Lancet*, 350:134-138, 1997.

Castano *et al.*, "*In vitro* formation of amyloid fibrils from two synthetic peptides of different lengths homologous to Alzheimer's disease beta-protein," *Biochem. Biophys. Res. Commun.*, 141(2):782-789, 1986.

Chang *et al.*, "Foreign gene delivery and expression in hepatocytes using a hepatitis B virus vector," *Hepatology*, 14:124A, 1991.

Chen and Okayama, "High-efficiency transfection of mammalian cells by plasmid DNA," *Mol. Cell Biol.*, 7:2745-2752, 1987.

Chesebro, "Transmissible spongiform encephalopathies: scrapie, BSE and related disorders," *Curr. Topics Microbiol. Immunol.*, Springer-Verlag, Berlin/Heidelberg,ed., vol. 172, 1991.

Choo, Schneider, Graciani, Kelly, *Macromol.*, 29:355-366, 1996.

Citron *et al.*, "Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases ú-protein production, *Nature*, 360:672-674, 1992 .

Coffin, "Retroviridae and their replication, "*In: Virology*, Fields BN, Knipe DM, ed., New York: Raven Press, pp. 1437-1500, 1990.

Cooper, *Lab. Invest.*, 31:232-238, 1974.

Couch *et al.*, "Immunization with types 4 and 7 adenovirus by selective infection of the intestinal tract," *Am. Rev. Resp. Dis.*, 88:394-403, 1963.

Coupar *et al.*, "A general method for the construction of recombinant vaccinia virus expressing multiple foreign genes," *Gene*, 68:1-10, 1988.

Crawford and Thiyagarajan, *The New Small Angle Diffractometer SAND at IPNS*, Proc. 13th International Collaboration on Advanced Neutron Sources, Villingen, Switzerland, Oct. 11-14, 1995, PSDI-Proc 95-02, 99, 1997.

Dubensky *et al.*, "Direct transfection of viral and plasmid DNA into the liver or spleen of mice," *Proc. Nat. Acad. Sci. USA,* 81:7529-7533, 1984.

Dudek and Johnson, "Transglutaminase facilitates the formation of polymers of the beta-amyloid peptide, *Brain Research*, 651:129-33, 1994.

Dyeing: Correlation of Histochemical and Chemical Data," *Histochemistry*, 77:431-445, 1983.

Esler, *et al.*, "Point substitution in the central hydrophobic cluster of human β-amyloid congener disrupts peptide folding and abolishes plaque competence," *Biochem.*, 35:13914-13921, 1996.

Esler, Stimson, Ghilardi, Lu, Felix, Vinters, Mantyh, Lee, Maggio, *Biochemistry*, 35:13914-13921, 1996.

Eyre *et al.*, "Cross-linking in collagen and elastin, *Ann. Rev. Biochem.*, 53:717-48, 1984.

Fabian *et al.*, "Comparative analysis of human and Dutch-type Alzheimer beta-amyloid peptides by infrared spectroscopy and circular dichroism. *Biochem. Biophys. Res. Commun.*, 191:232-239, 1993.

Fechheimer *et al.*, "Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading," *Proc. Natl. Acad. Sci. USA,* 84:8463-8467, 1987.

Ferkol *et al.*, "Regulation of the phosphoenolpyruvate carboxykinase/human factor IX gene introduced into the livers of adult rats by receptor-mediated gene transfer," *FASEB J.*, 7:1081-1091, 1993.

Fortier, "Biomedical applications of enzymes and their polyethylene glycol adducts. Biotech. Genet. Eng. Rev. 12:329-356, 1994.

Fraley *et al.*, "Entrapment of a bacterial plasmid in phospholipid vesicles: Potential for gene transfer," *Proc. Natl. Acad. Sci. USA,* 76:3348-3352, 1979.

Fraser, *et al.*, "pH-dependent structural transitions of Alzheimer amyloid peptides," *Biophys. J.*, 60:1190-1201, 1991.

Fraser, McLachlan, Surewicz, Mizzen, Snow, Nguyen, Kirschner, *J. Mol. Biol.*, 244:64-73, 1994.

Fraser, Nguyen, Inouye, Surewicz, Selkoe, Podlisny, Kirschner, *Biochemistry*, 31:10716-10723, 1992.

Freshner, "Animal Cell Culture: a Practical Approach," Second Edition, Oxford/New York, IRL Press, Oxford University Press, 1992.

Friedmann, "Progress toward human gene therapy," *Science*, 244:1275-1281, 1989.

Ghosh-Choudhury *et al.*, "Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full-length genomes," *EMBO J.*, 6:1733-1739, 1987.

Ghosh and Bachhawat, "Targeting of liposomes to hepatocytes," *In:* Wu G, Wu C ed. Liver diseases, targeted diagnosis and therapy using specific receptors and ligands, New York: Marcel Dekker, pp. 87-104, 1991.

Glenner and Wong, *Biochem. Biophys. Res. Commun.*, 122:1131-1135, 1984.

Glenner, Keiser, Bladen, Cuatrecasas, Eanes, Ram, Kanfer, DeLellis, *J. Histochem. Cytochem.*, 16:633-644, 1968.

Goate *et al.*, *Nature*, 349:704-706, 1991.

Goding, *In*: *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, Orlando, Fla., pp. 60-61 and 71-74, 1986.

Goldgaber, *Science*, 235:877-880, 1987.

Goldgaber, *Science*, 235:877-880, 1987.

Gomez-Foix *et al.*, "Adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen," *J. Biol. Chem.*, 267:25129-25134, 1992.

Gonzalez-Lima, Valla, Matos-Collazo, "Quantitative cytochemistry of cytochrome oxidase and cellular morphometry of the human inferior colliculus in control and Alzheimer's patients," *Brain Res.*, 752:117-126, 1997.

Gopal, "Gene transfer method for transient gene expression, stable transfection, and cotransfection of suspension cell cultures," *Mol. Cell Biol.*, 5:1188-1190, 1985.

Graham and Prevec, "Manipulation of adenovirus vector," *In*: *Methods in Molecular Biology: Gene Transfer and Expression Protocol*, E.J. Murray (ed.), Clifton, NJ: Humana Press, 7:109-128, 1991.

Graham and Prevec, "Adenovirus-based expression vectors and recombinant vaccines," *Biotechnology*, 20:363-390, 1992.

Graham and Van Der Eb, "A new technique for the assay of infectivity of human adenovirus 5 DNA," *Virology*, 52:456-467, 1973.

Graham *et al.*, "Characteristics of a human cell line transformed by DNA from human adenovirus type 5," *J. Gen. Virol.*, 36:59-72, 1977.

Greenfield and Fasman, "Computed circular dichroism spectra for the evaluation of protein conformation," *Biochem.*, 8:4108-4116, 1969.

Gregory *et al.*, "Double-quantum filtering in magic-angle-spinning NMR spectroscopy applied to DNA oligomers," *Molecular Physics.*, 89:1835-1849, 1996.

Gregory *et al.*, "Determination of local structure in solid nucleic acids using double quantum nuclear magnetic resonance spectroscopy," *J. Chem. Phys.*, 107:28-42, 1997.

Gregory, Mehta, Shiels, Drobny, *J. Chem. Phys.*, 107:28-42, 1997.

Grunhaus and Horwitz, "Adenovirus as cloning vector," *Seminar in Virology*, 3:237-252, 1992.

Gueft, *Mt. Sinai J. Med. N.Y.*, 39:91-102, 1972.

Haass *et al.*, "Amyloid beta-peptide is produced by cultured cells during normal metabolism, *Nature*, 359:322-325, 1992.

Han and Janda, *Angew. Chem., Int. Ed. Engl.*, 36:1731-33, 1997.

Hardy and Allsop, *Trends Pharmacol. Sci.*, 12:383-388, 1991.

Harlan *et al.*, *Nature*, 353:844-846, 1991.

Harland and Weintraub, "Translation of mammalian mRNA injected into Xenopus ocytes is specifically inhibited by antisense RNA," *J. Cell Biol.*, 101:1094-1099, 1985.

Harper, Lieber, Lansbury Jr., *Chem & Biol.*, 4:951-959, 1997.

Harris, M.J.(ed.) *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*, Plenum Press, New York, (1992).

Hermonat and Muzycska, *Proc. Nat. Acad. Sci. USA*, 81:6466-6470, 1984.

Hersdorffer *et al.*, "Efficient gene transfer in live mice using a unique retroviral packaging line," *DNA Cell Biol.*, 9:713-723, 1990.

Hershfield, "Enzyme replacement therapy of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase (PEG-ADA). *Immunodeficiency* 4:93-97, 1993.

Hershfield, "PEG-ADA replacement therapy for adenosine deaminase deficiency:an update after 8.5 years," *Clin. Immunol. Immunopathol.*, 76:S228-232, 1995.

Hershfield, *et al.*, "Enzyme replacement with polyethylene glycol-adenosine deaminase on adenosine deaminase deficiency:overview and case reports of three patients, including two now receiving gene therapy," *Peds. Res.*, 33:S42-S48, 1992.

Herz and Gerard, "Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice," *Proc. Nat'l. Acad. Sci. USA*, 90:2812-2816, 1993.

Higuchi *et al.*, *Biochemical Journal*. 279 (Pt 2):427-33, 1991.

Hilbich *et al.*, "Aggregation and secondary structure of synthetic amyloid beta A4 peptides of Alzheimer's disease," *J. Mol. Biol.*, 218(1):149-163, 1991.

Hope and Baybutt, "The key role of the membrane protein PrP in scrapie-like diseases," *Semin. Neurosci.*, 3:165-171, 1991.

Horwich and Weismann, "Deadly Conformations Protein in Prior Disease, *Cell*, 89:499-510, 1997.

Horwich and Weissman, "Deadly Conformations-Protein Misfolding in Prion Disease," *Cell*, 89:499-510, 1997.

Horwich *et al.* "Synthesis of hepadenovirus particles that contain replication-defective duck hepatitis B virus genomes in cultured HuH7 cells," *J. Virol.*, 64:642-650, 1990.

Ikura, *et al.*, "Cross-linking of a synthetic partial-length (1-28) peptide of the Alzheimer beta/A4 amyloid protein by transglutaminase, *FEBS Letters*, 326:109-111, 1993.

Ikura, *et al.*, *FFB*, 326:109, 1993.

Inada, *et al.*, "Biomedical and biotechnological applications of PEG- and PM-modified proteins," *Trends in Biotech.*, 13:86-91, 1995.

Inada, *et al.*, "Modification of proteins with polyethylene glycol derivatives, *Meth. Enzymol.*, 242:65-90, 1994.

Inada, Furukawa, Sasaki, Kodera, Hiroto, Nishimura, Matsushima, *Trends in Biotech.* 13:98-105, 1995.

Jacobson and Buxbaum, *Advances in Human Genetics*. 20:69-123, 309-11, 1991.

Jarrett and Lansbury, "Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?," *Cell*, 73:1055-1058, 1993.

Jarrett *et al.*, "Models of the β protein C terminus: Differences in amyloid structure lead to segregation of long and "short" fibril," *J. Am. Chem. Soc.*, 116:9741-9742, 1994.

Jarrett *et al.*, "The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease," *Biochem.*, 32:4693-4697, 1993.

Jayawickrama *et al.*, "Conformational analysis of the beta-amyloid peptide fragment, beta(12-28)," *J. Biomol. Struct. Dyn.*, 13:229-244, 1995.

Johnson *et al.*, *American Journal of Pathology*. 141(5):1013-9, 1992

Johnson *et al.*, Peptide Turn Mimetics" *In: Biotechnology And Pharmacy*, Pezzuto *et al.*, eds., Chapman and Hall, New York, 1993.

Jones and Shenk, "Isolation of deletion and substitution mutants of adenovirus type 5," *Cell*, 13:181-188, 1978.

Kaneda *et al.*, "Increased expression of DNA cointroduced with nuclear protein in adult rat liver," *Science*, 243:375-378, 1989.

Kang, *Nature*, 325:733-736, 1987.

Karlsson *et al.*, *EMBO J.*, 5:2377-2385, 1986.

Kato *et al.*, "Expression of hepatitis β virus surface antigen in adult rat liver," *J. Biol. Chem.*, 266:3361-3364, 1991.

Kaye *et al.*, *Journal of Clinical Pathology*. 39(5):581-2, 1986.

Kirschner *et al.*, "Synthetic peptide homologous to beta protein from Alzheimer disease forms amyloid-like fibrils *in vitro*," *Proc. Natl. Acad. Sci. USA*, 84(19):6953-6957, 1987.

Kirschner *et al.*, "X-ray diffraction from intraneuronal paired helical filaments and extraneuronal amyloid fibers in Alzheimer disease indicates cross-beta conformation, *Proc. Natl. Acad. Sci. USA*, 83:503-507, 1986.

Kirshenbaum and Daggett, "pH-dependent conformations of the amyloid beta(1-28) peptide fragment explored using molecular dynamics," *Biochem.*, 34:7629-7639, 1995.

Kisker, Schlindelin Alber, Ferry, Rees, *EMBO J.*, 15:2323-2330, 1996.

Kivela *et al.*, *Investigative Ophthalmology & Visual Science*. 35(10):3759-69, 1994.

Klein *et al.*, "High-velocity microprojectiles for delivering nucleic acids into living cells," *Nature*, 327:70-73, 1987.

Kodera, Nishimura, Matsushima, Hiroto, Inada, *J. Am. Oil. Chem.*, 71:335-8, 1994.

Kyte and Doolittle, "A simple method for displaying the hydropathic character of a protein," *J. Mol. Biol.*, 157(1):105-132, 1982.

Lansbury *et al.,* "Structural model for the P-amyloid fibril based on interstrand alignment of an antiparallel-sheet comprising a C-terminal peptide," *Nature Structural Biol.*, 2:990-998, 1995.

Lansbury Jr., Costa, Griffiths, Simon, Auger, Halverson, Kocisko, Hendsch, Ashburn, Spencer, Tidor, Griffin, *Nat. Struc. Biol.,* 2:990-998, 1995.

Lazo and Downing, *Biochemistry*, 37:1731-1735, 1998.

Le Gal La Salle *et al.*, "An adenovirus vector for gene transfer into neurons and glia in the brain," *Science*, 259:988-990, 1993.

Lee *et al*, "1H NMR of Aβ Amyloid Peptide Congeners in Water Solution. Conformational Changes Correlate with Plaque Competence," *Biochem.*, 34:5191-5200, 1995.

Levrero *et al.*, "Defective and nondefective adenovirus vectors for expressing foreign genes *in vitro* and *in vivo*," *Gene*, 101:195-202, 1991.

Li *et al.*, "Neuronal membrane conductance activated by amyloid beta peptide: importance of peptide conformation," *Brain Research.*, 682:207-211.

Liu and Parsons, *Macromolecules*, 2:529-33, 1969.

Lomakin, Chung, Benedek, Kirshner, Teplow, *Proc. Natl. Acad. Sci. USA*, 93:1125-1129, 1996.

Lomakin *et al.*, "On the nucleation and growth of amyloid β-protein fibrils:Detection of nuclei and quantitation of rate constants. *Proc. Natl. Acad. Sci. USA*, 93:1125-1129, 1996.

Lomakin *et al.*, "Title On the nucleation and growth of amyloid beta-protein fibrils: detection of nuclei and quantitation of rate constants," *Source Proceedings of the National Academy of Sciences of the United States of America,* 93(3):1125-9, Feb 6, 1996.

Malinchik, Inouye, Szumowski, Kirschner, *Biophys. J.*, 74:537-545, 1998.

Mann and Esiri, "The pattern of acquisition of plaques and tangles in the brains of patients under 50 years of age with Down's Syndrom," *J. Neurol. Sci.*, 1989.

Mann *et al.*, "Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus," *Cell*, 33:153-159, 1983.

Markowitz *et al.*, "A safe packaging line for gene transfer: Separating viral genes on two different plasmids," *J. Virol.*, 62:1120-1124, 1988.

Maxfield and Shepherd, *Polymer*, 16:505-9, 1975.

Merz *et al.*, "Ultrastructural morphology of amyloid fibrils from neuritic and amyloid plaques," *Acta Neuropathologica*, 60:113-124, 1983 .

Mirchev and Ferrone, "The structural link between polymerization and sickle cell disease. *J. Mol. Biol.*, 265:475-479, 1997.

Miroy *et al.*, "Inhibiting transthyretin amyloid fibril formation *via* protein stabilization," *Proc. Natl. Acad. Sci. USA*, 93:15051-15056, 1996.

Mullan *et al.*, *Nature Genet.*, 1:345-347, 1992.

Mulligan, "The Basic Science of Gene Therapy," *Science*, 260:926-932, 1993.

Murrell *et al.*, *Science*, 254:97-99, 1991.

Murrell *et al.*, "A Mutation in the Amyloid Precursor Protein associated with hereditary Alzheimer's disease," *Science*, 254:99-101, 1991.

Myers, EPO 0273085.

Narang, "High-resolution electron microscopic analysis of the amyloid fibril in Alzheimer's disease," *J. Neuropath. Exp. Neurol.*, 39:621-631, 1980.

Nesloney and Kelly, *Bioog. Med. Chem.*, 4:739-766, 1996.

Nicolas and Rubenstein, "Retroviral vectors," *In*: *Vectors: A survey of molecular cloning vectors and their uses*," Rodriguez and Denhardt (eds.), Stoneham: Butterworth, pp. 494-513, 1988.

Nicolau and Sene, "Liposome-mediated DNA transfer in eukaryotic cells," *Biochim. Biophys. Acta*, 721:185-190, 1982.

Nicolau *et al.*, "Liposomes as carriers for *in vivo* gene transfer and expression," *Methods Enzymol.*, 149:157-176, 1987.

Paskind *et al.*, "Dependence of moloney murine leukemia virus production on cell growth," *Virology*, 67:242-248, 1975.

Perales *et al.*, "Gene transfer *in vivo*: Sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake," *Proc. Natl. Acad. Sci.*, 91:4086-4090, 1994.

Porod, *In: Small Angle X-Ray Scattering*, Chapter 2, O.Glatter and O.Kratky, Eds., Academic Press, New York, NY, 1982.

Potter *et al.*, "Enhancer-dependent expression of human k immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation," *Proc. Nat'l Acad. Sci. USA*, 81:7161-7165, 1984.

Prusiner, "Molecular biology of prion diseases," *Science*, 252:1515-1522, 1991.

Pucci *et al.*, *Journal of Pathology*. 165(3):235-41, 1991.

Puchtler *et al.* "Application of Thiazole Dyes to Amyloid Under Conditions of Direct Cotton Dyeing: Correlation of Histochemical and Chemical Data," *Histochemistry*, 77:431-445, 1983.

Puchtler *et al.*, "On the binding of Congo Red by amyloid," *Cytochem.*, 10:355-364, 1962.

Racher *et al.*, *Biotechnology Techniques*, 9:169-174, 1995.

Rader *et al.*, *Journal of Lipid Research*. 33(5):755-63, 1992.

Ragot *et al.*, "Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of *mdx* mice," *Nature*, 361:647-650, 1993.

Rapp *et al.*, "Comparative Study of Antibody Titers Induced by a Peptide Epitope Conjugate with Protein, Lipopeptide, Polyoxyethylene and Polyoxyethylene-polystyrene Graft Copolymer," *In: Peptides 1990.* E. Girah and D. Andreu, ESCOM Science Publishers B.V., 1991.

Reiser *et al.*, "Enzymatic and nonenzymatic cross-linking of collagen and elastin," FASEB J. 6:2439-2449, 1992.

Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa.,16th ed., pp. 1035-1038 and 1570-1580, 1982.

Renan, "Cancer genes: Current status, future prospects and applications in radiotherapy/oncology," *Radiother. Oncol.*, 19:197-218, 1990.

Rich *et al.*, "Development and analysis of recombinant adenoviruses for gene therapy of cystic fibrosis," *Hum. Gene Ther.*, 4:461-476, 1993.

Ridgeway, "Mammalian expression vectors," *In:* Rodriguez RL, Denhardt DT, ed., Vectors: A survey of molecular cloning vectors and their uses, Stoneham: Butterworth, pp. 467-492, 1988.

Rippe *et al.*, "DNA-mediated gene transfer into adult rat hepatocytes in primary culture," *Mol. Cell Biol.*, 10:689-695, 1990.

Roher *et al*, "β-Amyloid from Alzheimer's disease brain inhibits spouting and survival of sympathetic neurons," *Biochem. Biophys. Res. Commun.*, 174:572-579, 1991.

Rosenfeld *et al.*, "Adenovirus-mediated transfer of a recombinant α1-antitrypsin gene to the lung epithelium *in vivo*," *Science*, 252:431-434, 1991.

Rosenfeld *et al.*, "*In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium," *Cell*, 68:143-155, 1992.

Rosser, "Molecular pathology of Alzheimer's Disease," *J. Neurol. Neurosurg. Psych.*, 56:583-586, 1993.

Rossler *et al.*, "Collagen microparticles: preparation and properties. *J. Microencapsulation*, 12:49-57, 1995.

Roux *et al.*, "A versatile and potentially general approach to the targeting of specific cell types by retroviruses: Application to the infection of human cells by means of major histocompatibility complex class I and class II antigens by mouse ecotropic murine leukemia virus-derived viruses," *Proc. Nat'l. Acad. Sci. USA*, 86:9079-9083, 1989.

Sato *et al.*, "Correlation among secondary structure, amyloid precursor protein accumulation, and neurotoxicity of amyloid beta(25-35) peptide as analyzed by single alanine substitution," *J. Biochem.*, 118:1108-1111, 1995.

Seilheimer, Bohrmann, Bondolfi, Muller, Stuber, Dobeli, *J. Struct. Biol.* 119, 59-71, 1997.

Selkoe, *Neuron* 6:487, 1991.

Selkoe, *J. Neuropathol. Exp. Neurol.*, 53:438-447, 1994.

Seubert, *et al.*," "Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids," *Nature*, 359:325-327, 1992.

Shen and Murphy, "Solvent effects on self-assembly of beta-amyloid peptide. *Biophysical Journal.*, 69(2):640-651, 1995.

Shoji, *et al.*, "Production of the Alzheimer amyloid beta protein by normal proteolytic processing," Science, 258:126-129, 1992..

Simmons, *et al.*, Secondary structure of amyloid beta peptide correlates with neurotoxic activity *in vitro*," *Mol. Pharmacol.*, 45:373-379, 1994.

Sipe, *Annu. Rev. Biochem.* 61:947-975, 1992.

Soregan, Kosmoski, Glabe, *J. Biol. Chem.* 269:28551-28554, 1994.

Soreghan, *et al.*, "Surfactant properties of Alzheimer's Aβ peptides and the Rajasekharan Pillai and Mutter, "Conformational studies of poly(oxyethylene)-bound peptide and protein sequences," *Acc. Chem. Res.*, 14:122-130, 1981.

Sormachi and Craik. "Structure determination of extracellular fragments of amyloid proteins involved in Alzheimer's disease and Dutch type hereditary cerebral haemorrhage with amyloidosis," *Eur. J. Biochem.*, 219:237-251, 1994.

Soto and Castano, "The conformation of Alzheimer's beta peptide determines the rate of amyloid formation and its resistance to proteolysis," *Biochem. J.*, 314:701-707, 1996.

Soto and Frangione, "Two conformational states of amyloid beta-peptide: implications for the pathogenesis of Alzheimer's disease," *Neurosci. Lett.*, 186:115-8, 1995.

Soto and Frangione, "Two conformational states of amyloid beta-peptide: implications for the pathogenesis of Alzheimer's disease," *Neuroscience Letters*, 186:115-118, 1995.

Soto, *et al*, "The α-helical to β-strand transition in the amino-terminal fragment of the amyloid β-peptide modulates amyloid formation. *J. Biol. Chem.*, 270:3063-3067, 1995.

Soutar *et al.*, *Proc. Nat'l Acad. Sci.* 89(16):7389-93, 1992.

Spera and Bax, "Empirical Correlation between Protein Backbone Conformation and Cα and Cβ 13C Nuclear Magnetic Resonance Chemical Shifts L," *Am. Chem. Soc.*, 113:5490-5492, 1991.

Steiner *et al.*, *Human Genetics*. 95(3):327-30, 1995.

Sticht, *et al.*, "Structure of amyloid A4-(1-40)-peptide of Alzheimer's disease," *Eur. J. Biochem.*, 233:293-298, 1995.

Stratford-Perricaudet and Perricaudet, *In: Human Gene Transfer*, Eds, O. Cohen-Haguenauer and M. Boiron, Editions John Libbey Eurotext, France, pp. 51-61, 1991.

Stratford-Perricaudet *et al.,* "Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector," *Hum. Gene. Ther.,* 1:241-256, 1990.

Talafous, *et al.*, "Solution structure of residues 1-28 of the amyloid beta-peptide," *Biochem.*, 33:7788-7796, 1994.

Tanzi, *Science*, 235:880-884, 1987.

Tanzi, *Science*, 235:880-884, 1987.

Temin, "Retrovirus vectors for gene transfer: Efficient integration into and expression of exogenous DNA in vertebrate cell genome," *In: Gene Transfer*, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188, 1986.

Teplow, *Int. J. Exp. Clin. Invest.*, 5:121-142, 1998.

Terzi *et al.*, "Reversible random coil-beta-sheet transition of the Alzheimer beta-amyloid fragment (25-35)," *Biochem.*, 33:1345-50, 1994.

Thiyagarajan, Chaiko, Hjelm Jr., *Macromolecules* 28:7730-7736, 1995.

Thiyagarajan, Epperson, Crawford, Carpenter, Kilppert, Wozniak, *J. Appl. Cryst.*, 30:280-293, 1997.

Tjernberg *et al.*,. Arrest of β-Amyloid fibril formation by a pentapeptide ligand, 1996.

Top *et al.*, "Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to adenovirus type 7," *J. Infect. Dis.*, 124:155-160, 1971.

Tur-Kaspa *et al.*, "Use of electroporation to introduce biologically active foreign genes into primary rat hepatocytes," *Mol. Cell Biol.*, 6:716-718, 1986.

U. S. Patent No. 4,196,265.

U. S. Patent No. 4,554,101.

U. S. Patent No. 4,666,829.

U. S. Patent No. 4,682,195.

U. S. Patent No. 4,683,202.

U. S. Patent No. 4,912,206.

U. S. Patent No. 5,474,893.

U. S. Patent No. 5,538,845.

U. S. Patent No. 5,643,562.

Varmus *et al.*, "Retroviruses as mutagens: Insertion and excision of a nontransforming provirus alter the expression of a resident transforming provirus," *Cell*, 25:23-36, 1981.

Virchow, *Virchows Arch.*, 8:140, 1854.

Wagner *et al., Proc. Natl. Acad. Sci.*, 87(9):3410-3414, 1990.

Weinberg, *et al.*, "T lymphocyte ontogeny in adenosine deaminase-deficient severe combined immunodeficiency after treatment with polyethylene glycol-modified adenosine deaminase. *J. Clin. Invest.*, 92:596-602, 1993.

Weinreb, *et al.*, "Peptide models of a hydrophobic cluster at the C-terminus of the β-amyloid protein," *J. Am. Chem. Soc.*, 116:10835-108361994, .

Wishart, *et al.*, "The Chemical Shift Index: A Fast and Simple Method for the Assignment of Protein secondary Structure through NMR Spectroscopy," *Biochemistry*, 31:1647-1651, 1992.

Wong *et al.*, "Appearance of β–lactamase activity in animal cells upon liposome mediated gene transfer," *Gene,* 10:87-94, 1980.

Wu and Wu, "Receptor-mediated *in vitro* gene transfections by a soluble DNA carrier system," *J. Biol. Chem.*, 262:4429-4432, 1987.

Wu and Wu, "Evidence for targeted gene delivery to HepG2 hepatoma cells *in vitro*," *Biochemistry*, 27:887-892, 1988.

Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.

Yamakazi, *et al.*, "Ultrastructural Characterization of cerebellar diffuse plaques in Alzheimer's disease," *J. Neuropath. Exp. Neurol.*, 51:281-286, 1992.

Yang *et al.*, "*In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment," *Proc. Natl. Acad. Sci USA,* 87:9568-9572, 1990.

Yankner, "Mechanisms of Neuronal Degeneration in Alzheimer's Disease," *Neuron*, 16:921-932, 1996.

Yankner, *et al.*, "Nerve growth factor potentiates the neurotoxicity of β amyloid," *Proc. Natl. Acad. Sci. USA*, 87:9020-9023, 1990.

Yankner, *et al.*, "Neurotoxicity of the amyloid Precursor Associated with Alzheimer's Disease, *Science*, 245:417-429, 1989.

Yankner, *et al.*, "Neurotrophic and neurotoxic effects of amyloid β protein:reversal by tachykinin neuropeptides," *Science*, 250:279-282, 1990.

Ye, Fu, Hu, Hou, Ding, *Magn. Reson. Chem.*, 31:699-704, 1993.

Zagorski and Barrow, "NMR studies of amyloid ú peptides: proton assignments, sheet conversion for a homologous, 28-residue, N-terminal fragment," *Biochem.*, 31:5621-5631, 1992.

Zelenin *et al.*, "High-velocity mechanical DNA transfer of the chloramphenicol acetyltransferase gene into rodent liver, kidney and mammary gland cells in organ explants and *in vivo*," *FEBS Lett.*, 280:94-96, 1991.

CLAIMS:

1. A method of inhibiting amyloid fibrillogenesis comprising contacting tissue with a composition comprising an amyloidogenic peptide that has been blocked.

2. The method of claim 1, wherein said peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide.

3. The method of claim 1, wherein said peptide is blocked at the C-terminus of the peptide.

4. The method of claim 1, wherein said peptide is blocked at a side chain of the peptide.

5. The method of claim 1, wherein said peptide is blocked using a polymer.

6. The method of claim 5, wherein said polymer is polyethylene glycol.

7. The method of claim 1, wherein said amyloidogenic peptide is selected from the group consisting of cystatin C, AApoA-I, AApoA-II, AScr or PrP-27, β-amyloid peptide, amyloid A, amyloid kappa L-chain, amyloid lambda L-chain, A β2M, ATTR, AIAPP, amylin atrial naturetic factor, procalcitonin and gelsolin.

8. The method of claim 1, wherein said tissue is selected from the group consisting of pancreas, central nervous system, peripheral nervous system, muscle and heart.

9. The method of claim 8, wherein said central nervous system tissue is further defined as brain tissue.

10. The method of claim 1, wherein said tissue is from a subject having a pathological state selected from the group consisting of Alzheimer's Disease, Down's Syndrome, Dutch-Type Hereditary Cerebral Hemorrhage Amyloidosis, Reactive Amyloidosis, Familial Mediterranean Fever, Familial Amyloid Nephropathy With Urticaria And Deafness, Muckle-Wells Syndrome, Idiopathic Myeloma, Macroglobulinemia-Associated Myeloma, Familial Amyloid Polyneuropathy, Familial Amyloid Cardiomyopathy, Isolated Cardiac Amyloid, Systemic Senile Amyloidosis, Adult Onset Diabetes, Insulinoma, Isolated Atrial Amyloid, Medullary Carcinoma Of The Thyroid, Familial Amyloidosis, Hereditary Cerebral Hemorrhage With Amyloidosis, Familial Amyloidotic Polyneuropathy, Scrapie, Creutzfeldt-Jacob Disease, Gerstmann-Straussler-Scheinker Syndrome and Bovine Spongiform Encephalitis.

11. The method of claim 10, wherein said subject is a human.

12. A method of inhibiting amyloid fibrillogenesis comprising contacting tissue with a composition comprising a β–amyloid peptide that has been blocked at an end terminal or a side chain.

13. The method of claim 12, wherein said peptide is blocked using a polymer.

14. The method of claim 13, wherein said polymer is polyethylene glycol.

15. The method of claim 12, wherein said β–amyloid peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide.

16. The method of claim 15, wherein said β–amyloid peptide is blocked at the C-terminus of the peptide.

17. The method of claim 15, wherein said β–amyloid peptide is blocked at a side chain of the peptide.

18. The method of claim 12, wherein said β–amyloid peptide is a full length β–amyloid peptide.

19. The method of claim 12, wherein said β–amyloid peptide comprises a partial sequence of a full length β–amyloid peptide.

20. The method of claim 12, wherein said β–amyloid peptide comprises a peptide having amino acid residues 10 through to 35 β–amyloid peptide.

21. The method of claim 12, wherein said β–amyloid peptide has the sequence of SEQ ID NO:1.

22. The method of claim 12, wherein said β–amyloid peptide has the sequence of SEQ ID NO:2.

23. The method of claim 14, wherein said polyethylene glycol has a molecular weight of between about 1000 and 6000 Da.

24. The method of claim 23, wherein said polyethylene glycol has a molecular weight of about 3000 Da.

25. A β–amyloid peptide that is blocked by conjugation to polyethylene glycol.

26. The β–amyloid peptide of claim 25, wherein said β-amyloid peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide.

27. The β–amyloid peptide of claim 26, wherein said β–amyloid peptide is blocked at the C-terminus of the peptide.

28. The β–amyloid peptide of claim 26, wherein said β–amyloid peptide is blocked at a side chain of the peptide.

29. The β–amyloid peptide of claim 26, wherein said β–amyloid peptide is a full length β–amyloid peptide.

30. The β–amyloid peptide of claim 26, wherein said β–amyloid peptide comprises a peptide having amino acid residues 10 through to 35 β–amyloid peptide.

31. The β–amyloid peptide of claim 26, wherein said β–amyloid peptide has the sequence of SEQ ID NO:1.

32. The β–amyloid peptide of claim 26, wherein said β–amyloid peptide has the sequence of SEQ ID NO:2.

33. The β–amyloid peptide of claim 26 wherein said polyethylene glycol has a molecular weight of about 3000.

34. A method of inhibiting amyloid plaque formation in a subject comprising administering a pharmaceutical composition comprising a β–amyloid peptide blocked by conjugation to a second compound and a pharmaceutically acceptable buffer, solvent or diluent.

35. The method of claim 34, wherein said second compound is blocked using a polymer.

36. The method of claim 35, wherein said polymer is polyethylene glycol.

37. The method of claim 36, wherein said β–amyloid peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide.

38. The method of claim 37, wherein said β–amyloid peptide is blocked at the C-terminus of the peptide.

39. The method of claim 37, wherein said β–amyloid peptide is blocked at a side chain of the peptide.

40. The method of claim 34, wherein said administering is effected by regional delivery of the pharmaceutical composition.

41. The method of claim 34, wherein said administering comprises delivering said pharmaceutical composition endoscopically, intratracheally, percutaneously, or subcutaneously.

42. The method of claim 34, wherein said subject is a mammal.

43. The method of claim 42, wherein said subject is a human.

44. The method of claim 34, wherein said subject suffers from amyloidosis.

45. The method of claim 34, wherein said subject has Alzheimer's disease.

46. The method of claim 34, wherein said subject has Down's Syndrome.

47. A pharmaceutical composition comprising a β–amyloid peptide blocked by conjugation to a second compound and a pharmaceutically acceptable buffer, solvent or diluent.

48. The pharmaceutical composition of claim 47, wherein said second compound is a polymer.

49. The pharmaceutical composition of claim 48, wherein said polymer is polyethylene glycol.

50. The pharmaceutical composition of claim 47, wherein said β–amyloid peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide.

51. A β–amyloid peptide blocked by conjugation to a second compound.

52. The β–amyloid peptide of claim 51, wherein said second compound is a polymer.

53. The β–amyloid peptide of claim 52, wherein said polymer is polyethylene glycol.

54. The β–amyloid peptide of claim 53, wherein said β–amyloid peptide is blocked at one or more sites selected from the group consisting of the C-terminus, the N-terminus and a side chain of the peptide.

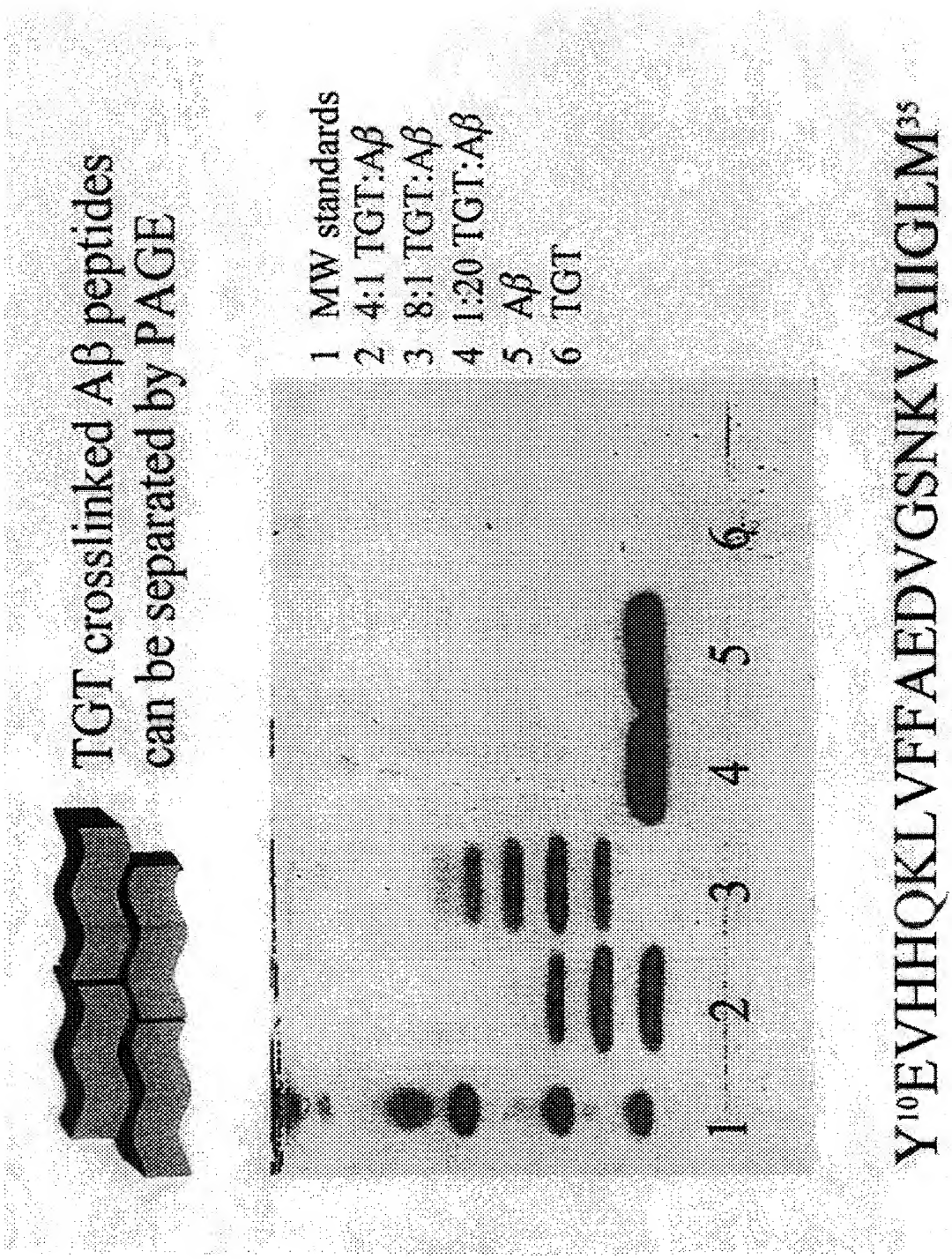

TGT crosslinked Aβ peptides
can be separated by PAGE
1 MW standards
2 4:1 TGT:Aβ
3 8:1 TGT:Aβ
4 1:20 TGT:Aβ
5 Aβ
6 TGT
1 2 3 4 5 6
Y10EVHHQKLVFFAEDVGSNKVAIIGLM35

FIG. 1

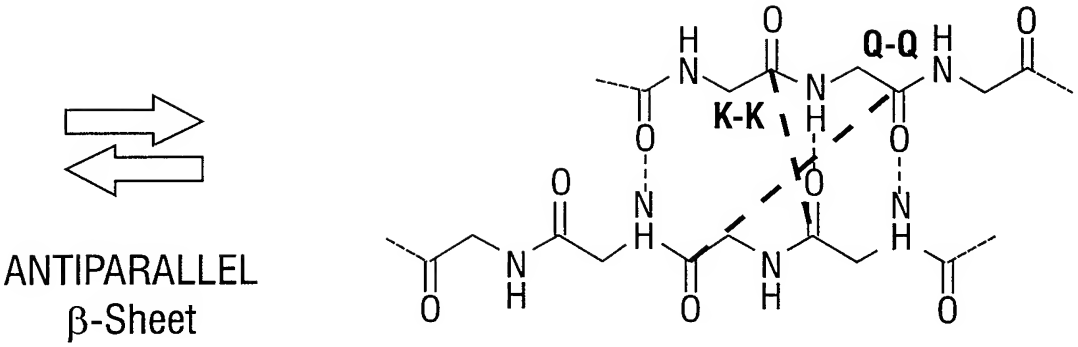


FIG. 2A

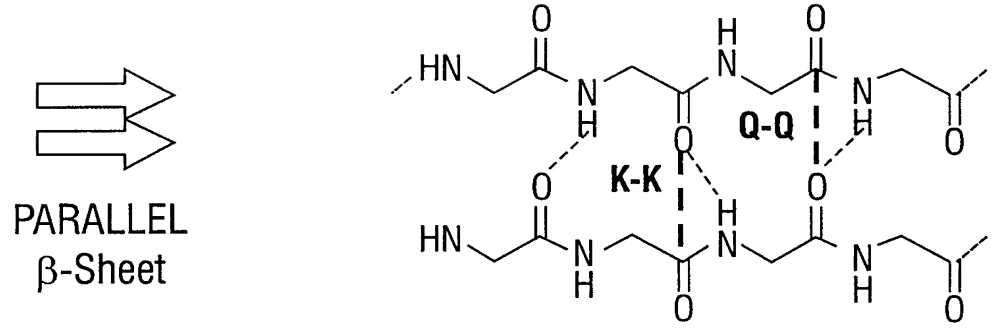


FIG. 2B

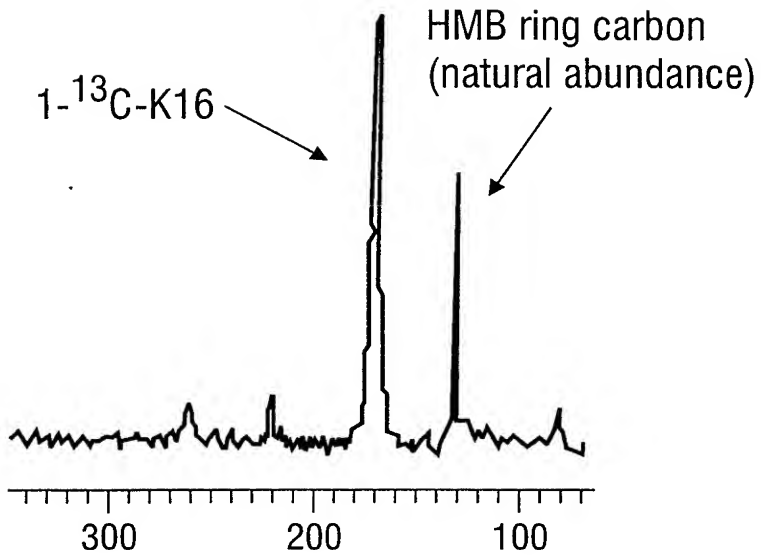

HMB ring carbon
(natural abundance)
1-13C-K16
300
200
100

FIG. 3A

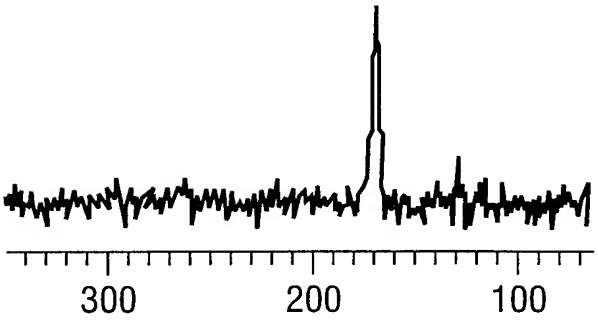

300
200
100

FIG. 3B

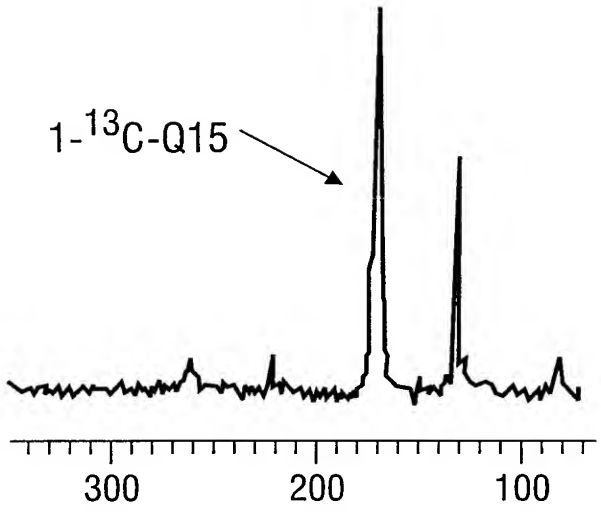


FIG. 3C

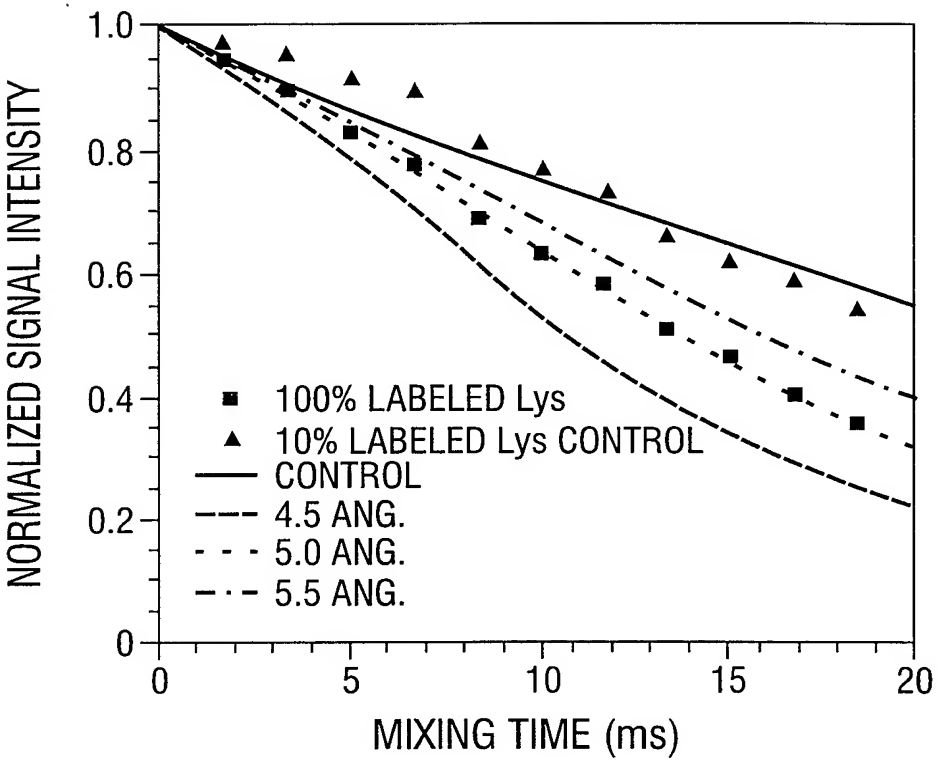

NORMALIZED SIGNAL INTENSITY
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
MIXING TIME (ms)
100% LABELED Lys
10% LABELED Lys CONTROL
CONTROL
4.5 ANG.
5.0 ANG.
5.5 ANG.

FIG. 4

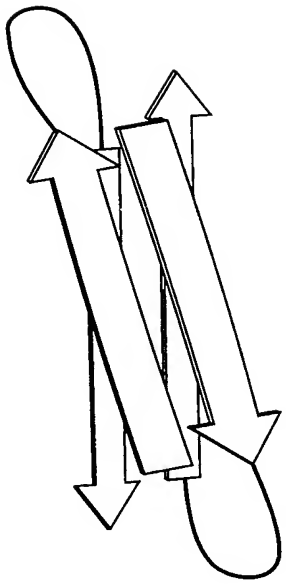


FIG. 5A

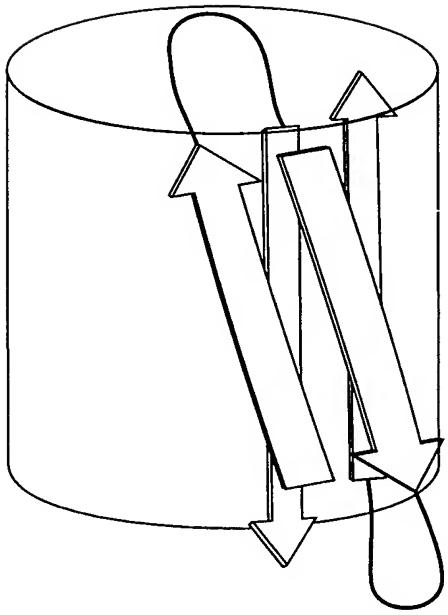


FIG. 5B

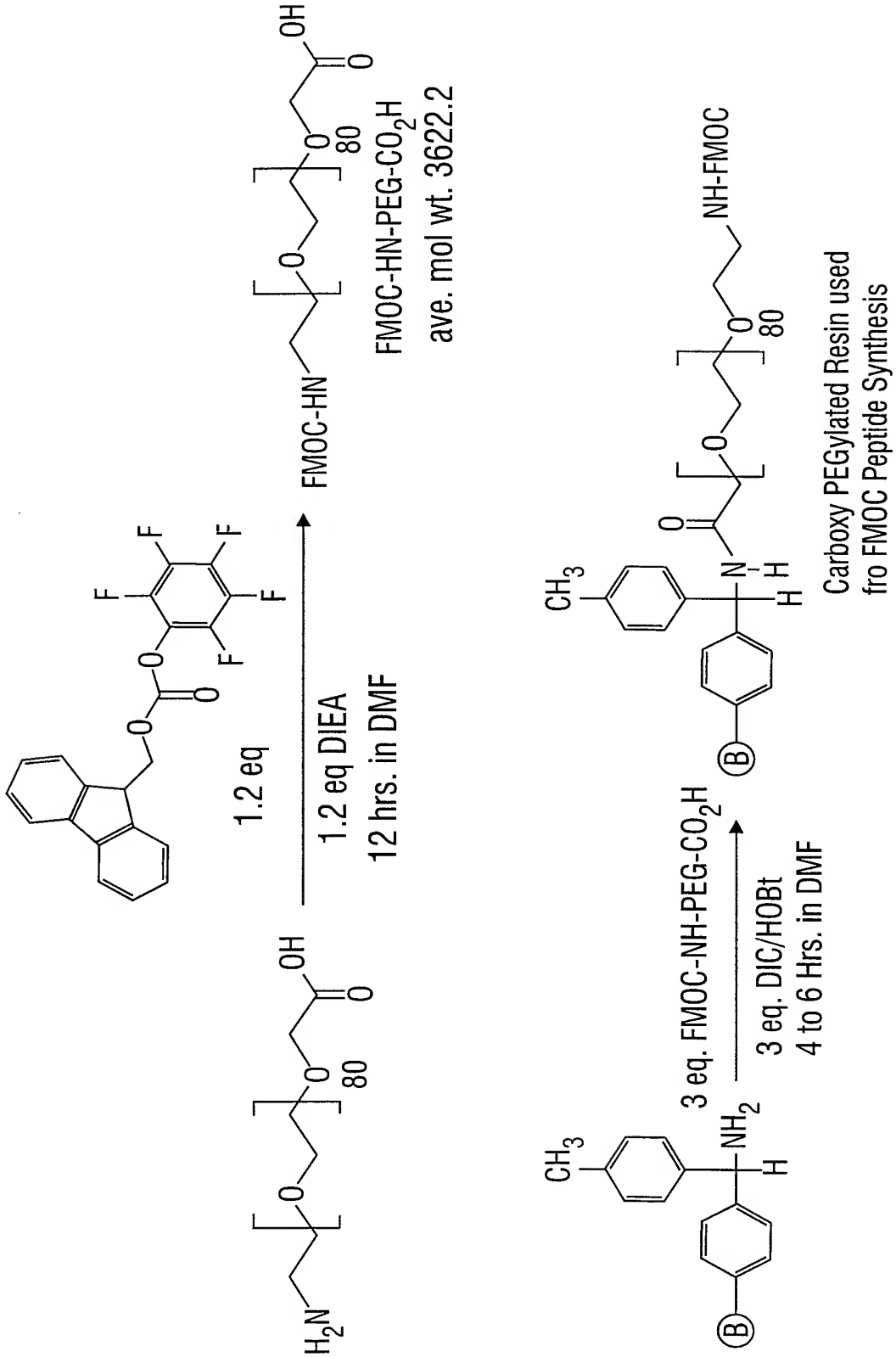

1.2 eq
1.2 eq DIEA
12 hrs. in DMF
FMOC-HN-PEG-CO2H
ave. mol wt. 3622.2
3 eq. FMOC-NH-PEG-CO2H
3 eq. DIC/HOBt
4 to 6 Hrs. in DMF
Carboxy PEGylated Resin used
fro FMOC Peptide Synthesis

FIG. 6

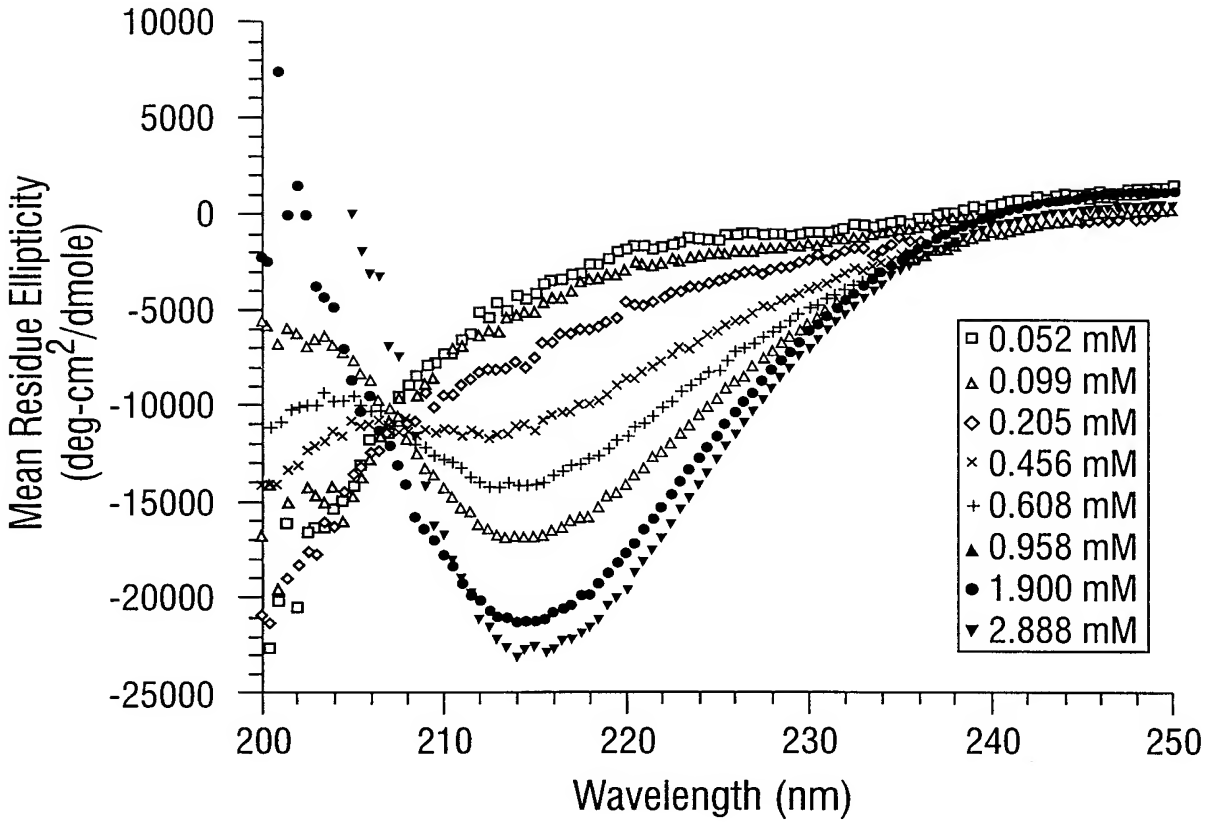

10000
5000
0
-5000
-10000
-15000
-20000
-25000
Mean Residue Ellipticity
(deg-cm2/dmole)
200
210
220
230
240
250
Wavelength (nm)
0.052 mM
0.099 mM
0.205 mM
0.456 mM
0.608 mM
0.958 mM
1.900 mM
2.888 mM

FIG. 7A

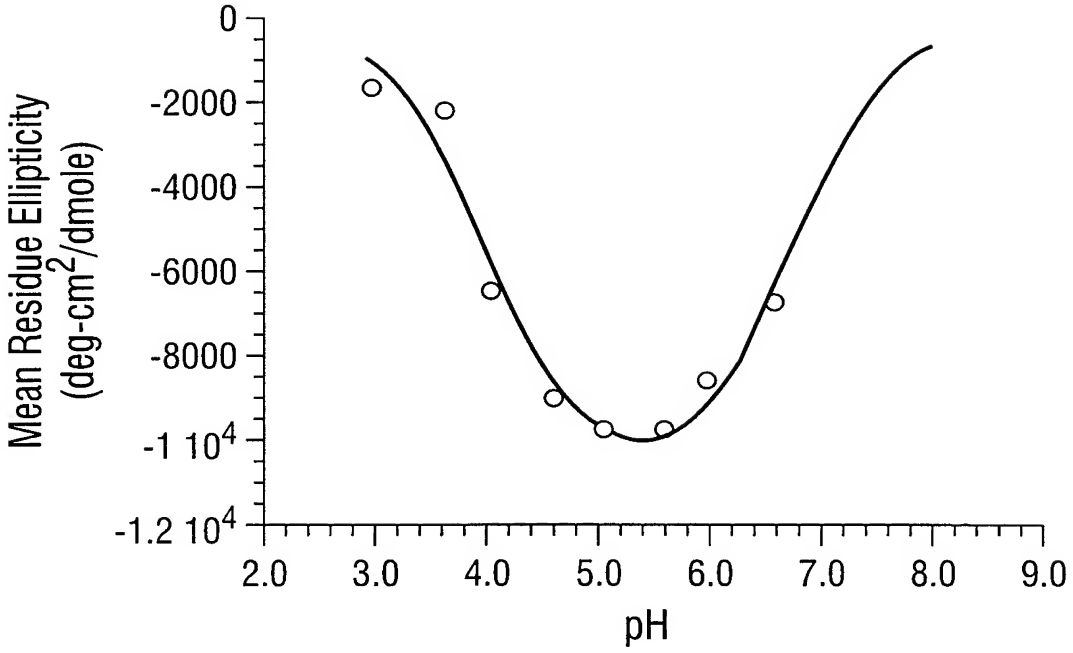

Mean Residue Ellipticity
(deg-cm^2/dmole)
0
-2000
-4000
-6000
-8000
-1 10^4
-1.2 10^4
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH

FIG. 7B

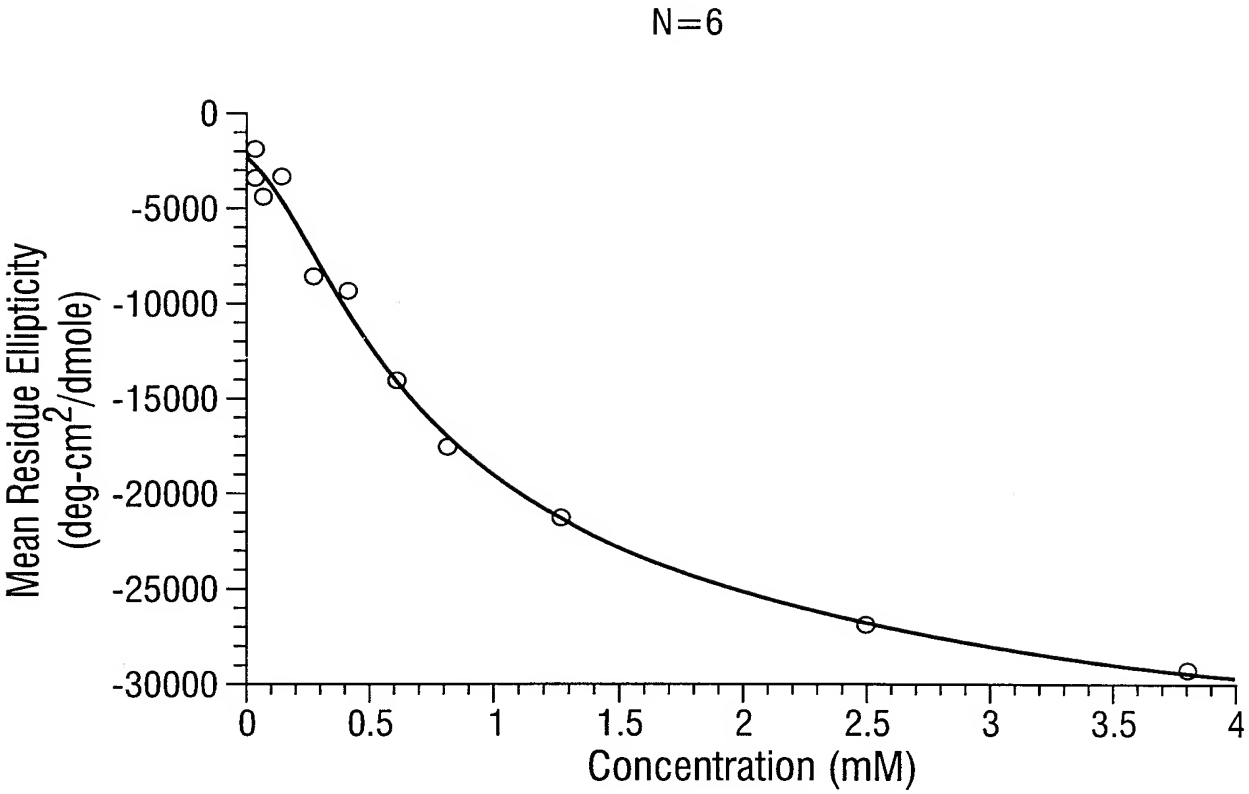

N=6
Mean Residue Ellipticity
(deg-cm2/dmole)
0
-5000
-10000
-15000
-20000
-25000
-30000
0
0.5
1
1.5
2
2.5
3
3.5
4
Concentration (mM)

FIG. 7C

^{1}DAEFRHDSG^{10}YEVHHQK**LVFFA**EDVGSNK**GAIIGL**^{35}**MVGGVVI**^{42}**A**

Aβ $_{(10-35)}$ ^{10}YEVHHQK**LVFFA**EDGSNK**GAIIGL**^{35}**M**

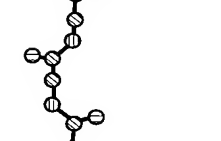
Aβ-PEG ^{10}YEVHHQK**LVFFA**EDGSNK**GAIIGL**^{35}**M** 

FIG. 8A

FIG. 8B

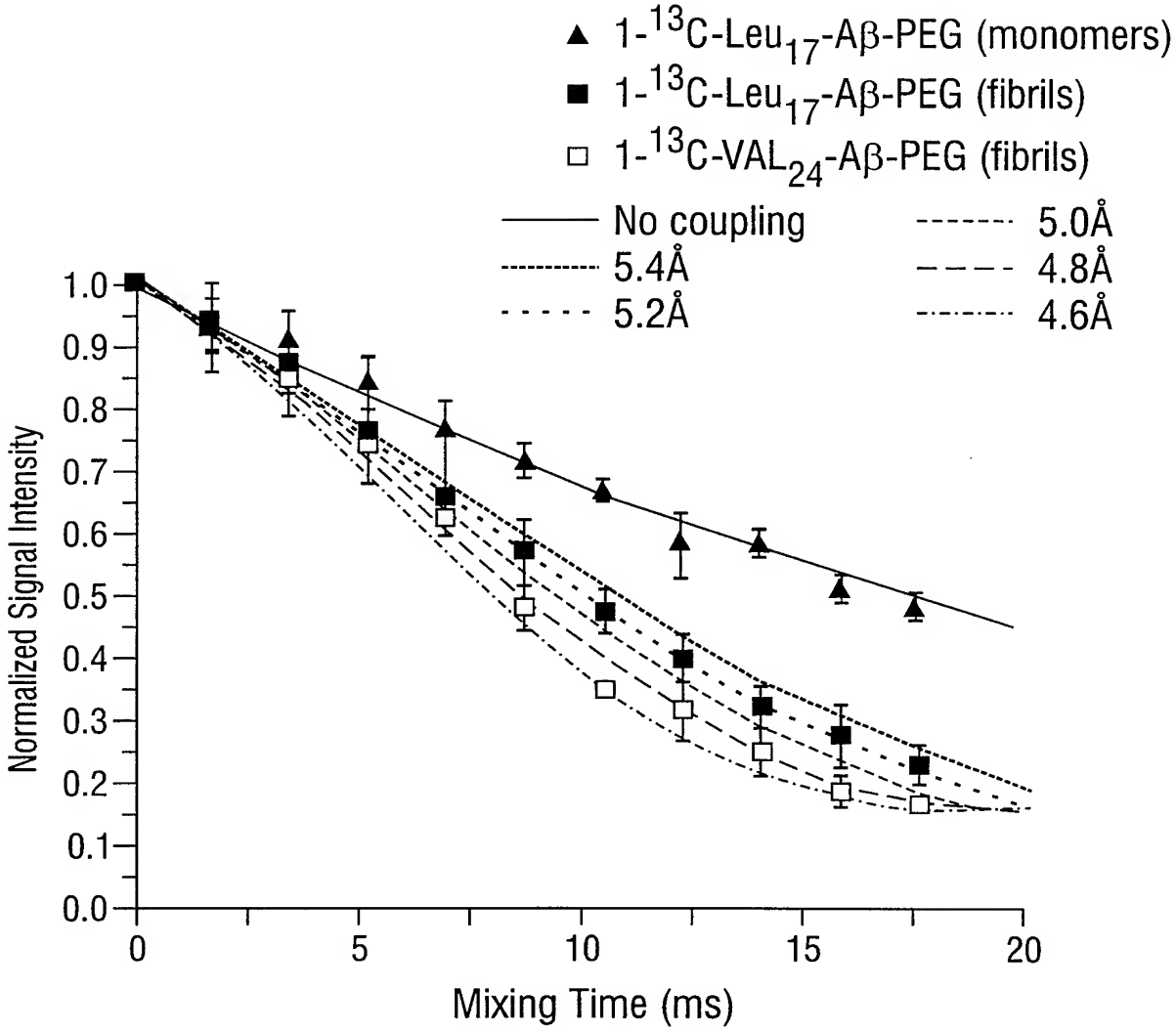

▲ 1-13C-Leu17-Aβ-PEG (monomers)
■ 1-13C-Leu17-Aβ-PEG (fibrils)
□ 1-13C-VAL24-Aβ-PEG (fibrils)
No coupling
5.4Å
5.2Å
5.0Å
4.8Å
4.6Å
Normalized Signal Intensity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
Mixing Time (ms)

FIG. 8C

FIG. 9A

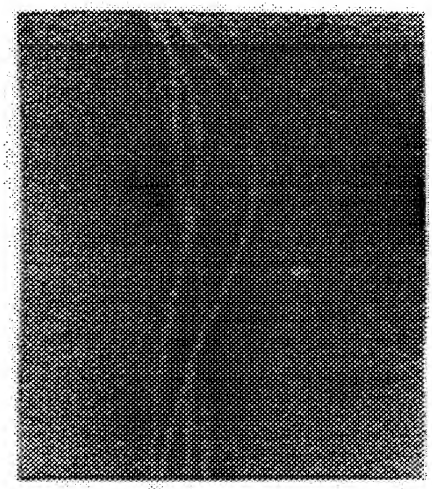


FIG. 9B

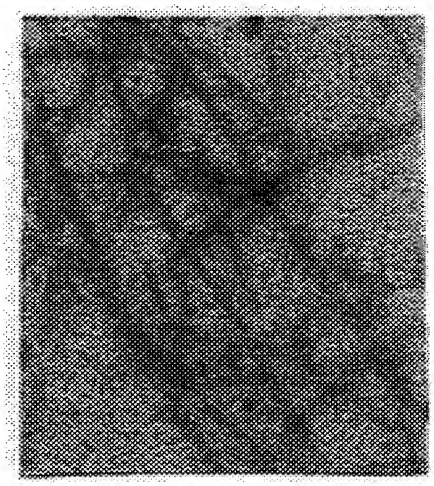


FIG. 9C

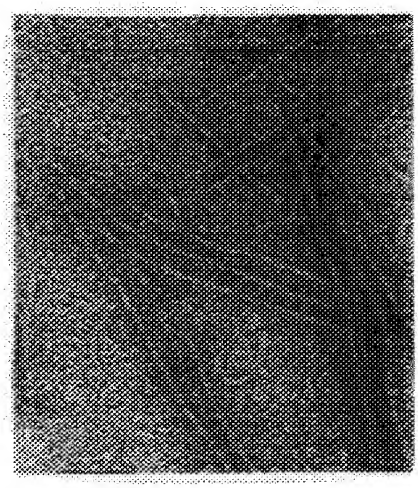


FIG. 9D

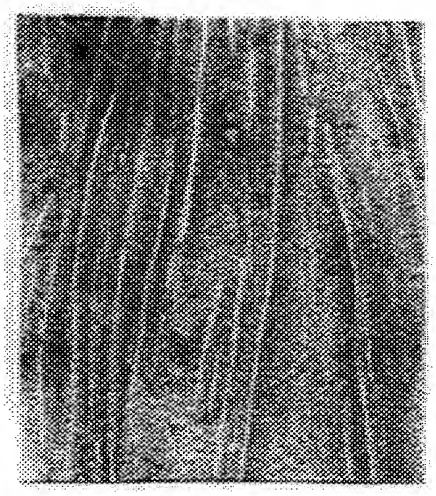


FIG. 9E

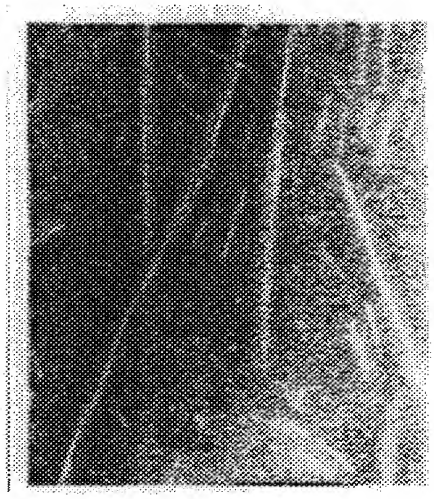


FIG. 9F

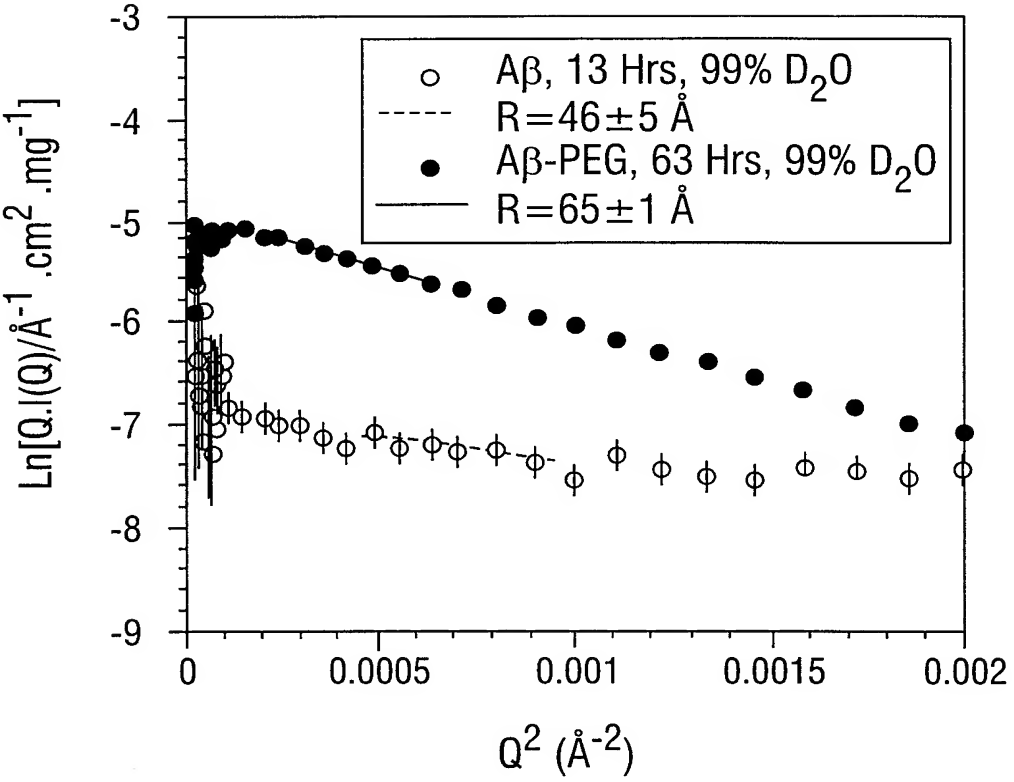


FIG. 10A

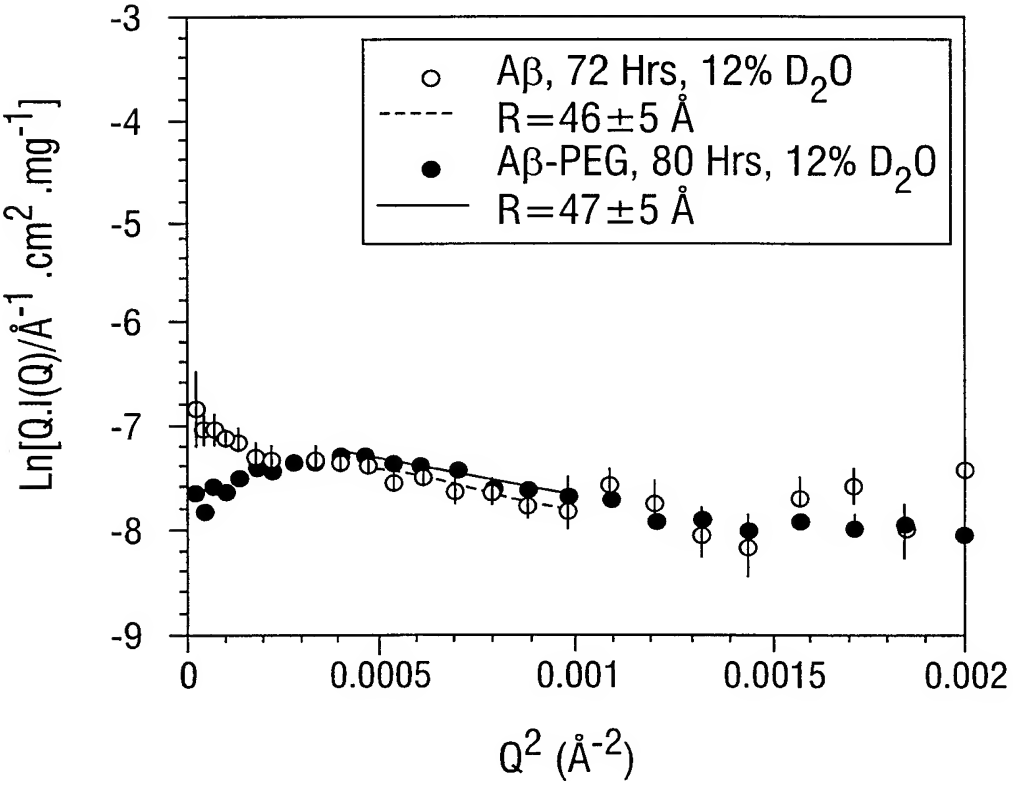


FIG. 10B

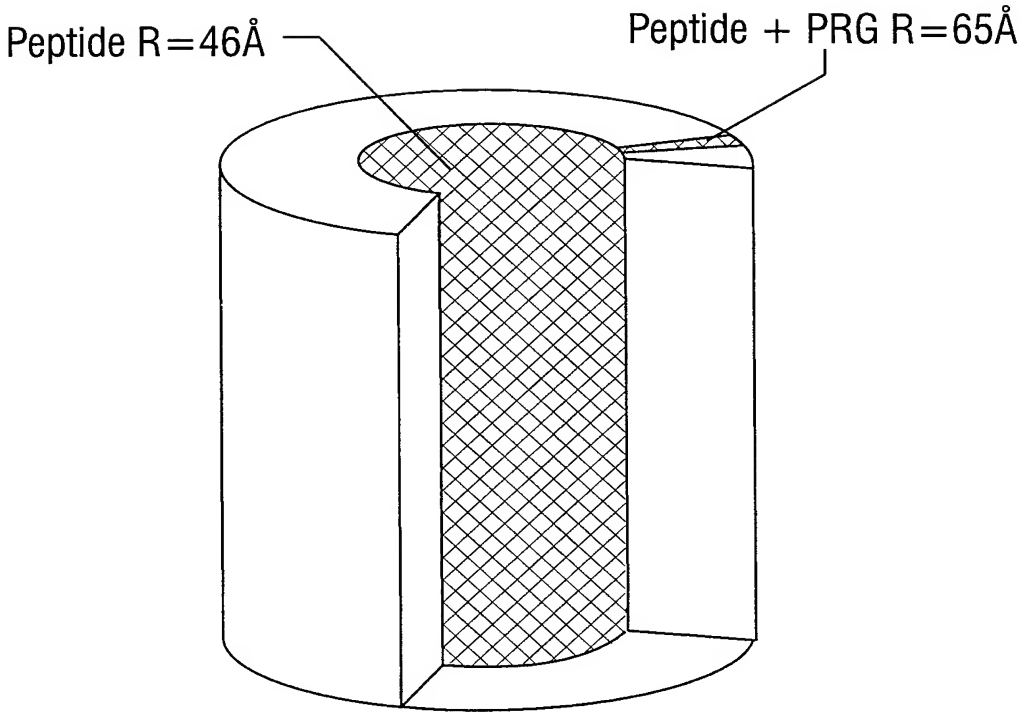


FIG. 10C

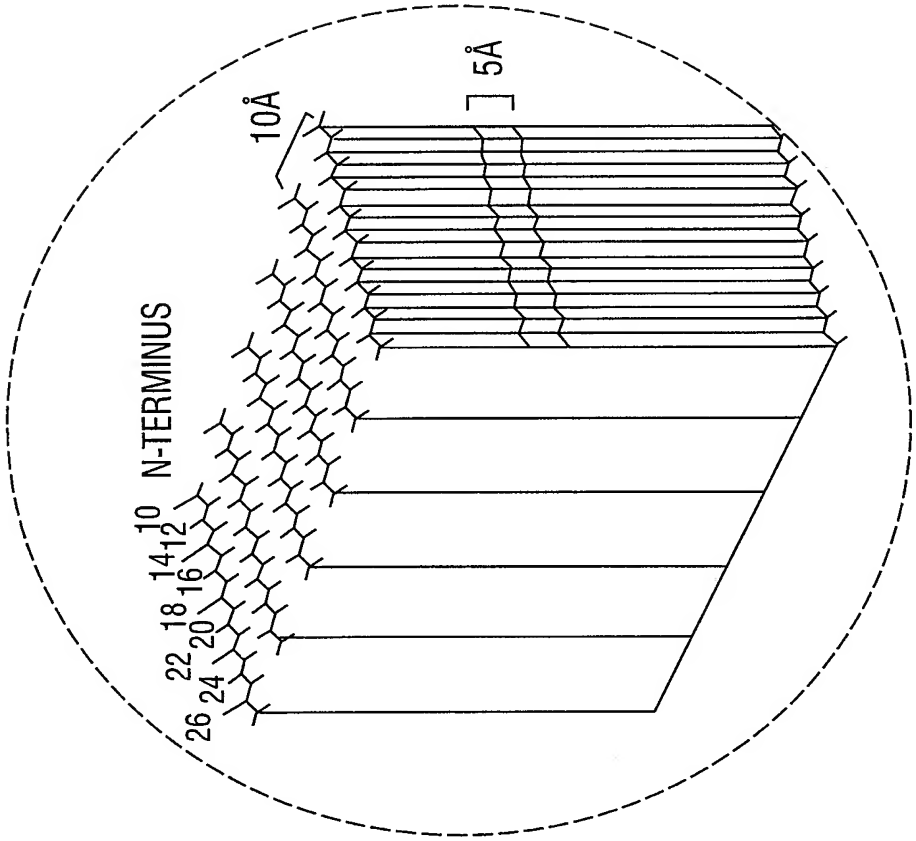


FIG. 11D

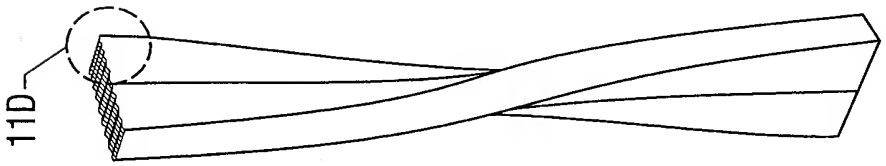


FIG. 11C

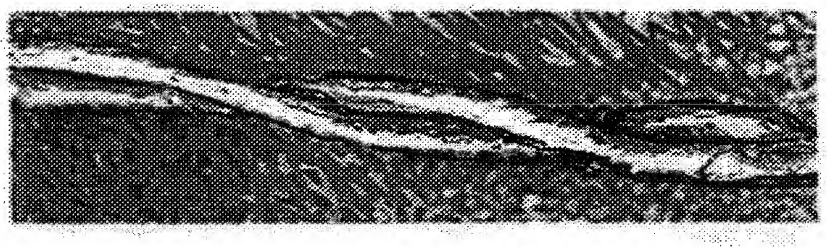


FIG. 11B

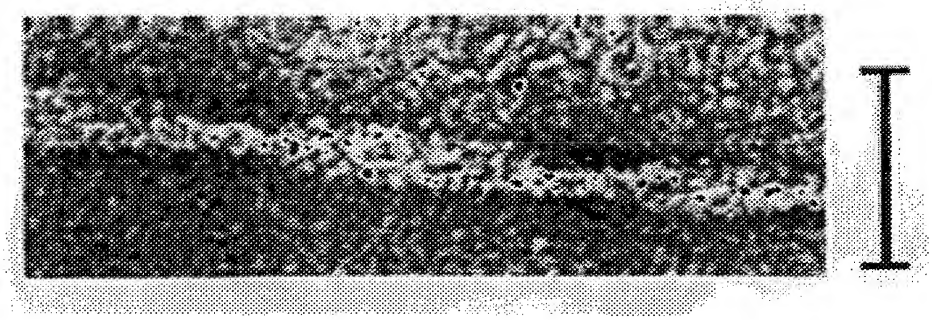


FIG. 11A

SEQUENCE LISTING

<110> LYNN, DAVID G.
MEREDITH, STEPHEN C.
BURKOTH, TIMOTHY S.

<120> METHODS AND COMPOSITIONS COMPRISING THE USE OF BLOCKED B-AMYLOID PEPTIDE

<130> ARCD:312/ARCD312P

<140> UNKNOWN
<141> 1999-02-12

<150> 60,074,658
<151> 1998-02-13

<160> 3

<170> PatentIn Ver. 2.0

<210> 1
<211> 42
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

<400> 1
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1 5 10 15

Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
20 25 30

Gly Leu Met Val Gly Gly Val Val Ile Ala
35 40

<210> 2
<211> 26
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

<400> 2
Tyr Glu Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly
1 5 10 15

Ser Asn Lys Gly Ala Ile Ile Gly Leu Met
20 25

<210> 3
<211> 27
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

<400> 3
His Tyr Glu Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val
1 5 10 15

Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met
20 25